THE FORMATION OF AN AZO ANION FREE RADICAL METABOLITE DURING THE MICROSOMAL AZO REDUCTION OF SULFONAZO III

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SUMMARY: An ESR spectrum is observed during the anaerobic incubation of the diazonaphthol dye sulfonazo III, with rat hepatic microsomes and NADPH. This spectrum is characterized by a partially resolved 17-line hyperfine pattern and g = 2.0034, as is consistent with the spectrum of an azo anion free radical, $[R\text{-}N\text{-}N\text{-}R']^{\cdot-}$. Oxygen, which strongly inhibits microsomal azoreductase, destroys the ESR signal. The oxidation of the azo anion radical metabolite by oxygen to the parent azo dye may account for the oxygen inhibition of microsomal azoreductase.

Introduction: We have previously reported that the enzymatic reduction of nitro drugs, nitro carcinogens, and other nitro compounds, gives nitro anion free radicals (1-3). In view of the many similarities of the microsomal azo- and nitroreductase, we proposed that the first intermediate of azo reduction is also a free radical metabolite, the azo anion free radical $[R\text{-}N\text{-}N\text{-}R']^{\cdot-}$ (2). Previous investigations of microsomal azoreductase have only considered the formation of the hydrazo intermediates or the amine cleavage products, presumably by a hydride transfer (4-6).

$$R\text{-}N{=}N\text{-}R' \xrightarrow[H^+]{H^-} R\text{-}NH\text{-}NH\text{-}R' \xrightarrow[H^+]{H^-} R\text{-}NH_2 + R'\text{-}NH_2$$

We now present evidence for the formation of a one-electron reduction metabolite in microsomal incubations containing sulfonazo III.

$$R\text{-}N{=}N\text{-}R' \xrightarrow{e^-} [R\text{-}N\text{-}N\text{-}R']^{\cdot-}$$

The oxygen inhibition of microsomal azoreductase may be caused by the air oxidation of this radical metabolite rather than the air oxidation of the hydrazo intermediate, as previously suggested (7).

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Table I. g-Values of the Sulfonazo III Radical Metabolite and Other Nitrogen Containing Organic Radicals

Radical	Isotropic g-Value[a]	Reference
Sulfonazo III radical metabolite	2.0034	this work
Azobenzene anion radicals (t-butyl substituted)	2.0036	11
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	2.00354	12
Diphenylnitroxide	2.0055	13
Nitrobenzene anion radical	2.0049	14
Wurster's blue cation (N,N,N',N'-tetramethyl-*p*-phenylenediamine cation radical)	2.003051	15

[a]Not corrected for second order shifts

Methods: Preparation of rat hepatic microsomes and the anaerobic incubation conditions have been previously described (2). The g-value of the sulfonazo III free radical metabolite was determined relative to the g-value of solid 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Fremy's salt (0.1 mM in 0.05 M K_2CO_3) using a Varian E-4 spectrometer. A capillary tube containing one of the secondary g-value standards was attached to an aqueous flat cell containing the microsomal incubation. Spectra with and without the g-value standards were taken. The calculation of the g-value of sulfonazo III was done using the equation:

$$g = g_{standard} [1 - (\Delta H/H_{standard})].$$

The magnetic field at the center of the ESR spectra of the respective standards, $H_{standard}$, was determined from the field dial. The azo free radical metabolite has its center at a higher field than DPPH (g = 2.0037 ± 0.0002) (8) or Fremy's salt (g = 2.00550 ± 0.00005) (9). This corresponds to a smaller g-value and a positive field separation, ΔH. The magnetic field sweep was calibrated with the known splitting constant of Fremy's salt (a_N = 13.091 ± 0.004 G) (10).

The disappearance of sulfonazo III was followed at the spectral maximum of 573 nm in a Gilford 2000 recording spectrophotometer. The absorption difference was determined between sulfonazo III and the microsomal reduction product as $\varepsilon = 1.91 \times 10^4$ $cm^{-1}M^{-1}$.

Results: The g-value of sulfonazo III is 2.0034 relative to either solid DPPH or Fremy's salt, with a probable experimental error of ± 0.0002 (Table I). This g-value is indistinguishable from the g-values of the azobenzene anion free radical (11) or the chemically related DPPH (12). This result suggests that the

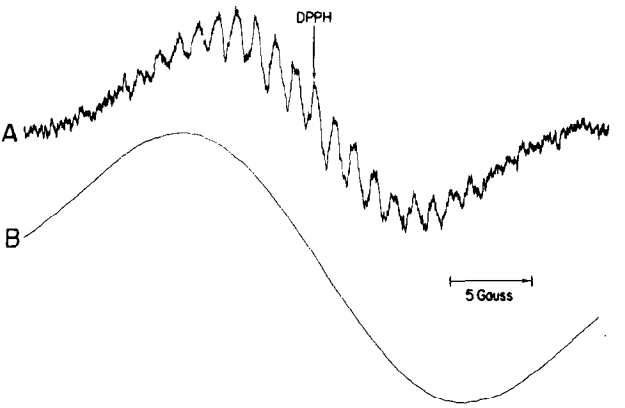
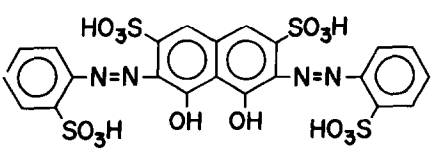


Fig. 1. The spectrum of the free radical formed in the first microsomal incubation of Table 2 is shown in A with the instrumental settings which gave the best resolution. The nominal microwave power was 0.5 mW and the modulation amplitude was 0.8 G. Eight partially resolved lines are downfield and eight are upfield of the central line. The DPPH marker is just downfield of the central line. In B the spectrum of the same sample is shown under conditions which maximize the signal-to-noise ratio. The nominal microwave power was 5.0 mW and the modulation amplitude was 20 G.

sulfonazo III free radical metabolite is in fact an azo anion radical, $[R\text{-}N\text{-}N\text{-}R']^{\cdot}$ Unfortunately, the g-values of most free radicals are within 2% of the free electron value of 2.0023, and a coincidental equality of g-values must be considered. For comparison, a second group of organic radicals is included in Table I. These radicals have spin density localized on a nitrogen atom, but are not chemically related to the azo anion free radicals. Note that Wurster's blue cation has a g-value similar to, but significantly different from, that of the sulfonazo III radical metabolite.

The fact that the spectrum in Figure 1A has a central line implies that all of the protons of the free radical occur in magnetically equivalent pairs, as is consistent with the structure of the radical precursor.

(figure of chemical structure)



Since the azobenzene anion free radical has a pK_a 7.1 (16), the sulfonazo III

Table II. The Effect of Atmospheric Conditions on the Disappearance of Sulfonazo III in Rat Hepatic Microsomal Incubations

Atmosphere	Dye Disappearance[a] (nmoles/min-mg protein)	Relative Activity
N_2	58.5 ± 1.0	100
Air	1.7 ± 0.1	2.9
CO	51.1 ± 0.8	87.4
N_2 (microsomes-heated 57° for 15 min.)	1.7 ± 0.1	2.9
N_2 (no microsomes)	1.4 ± 0.1	2.4
Air (no microsomes 8.0 mM NADPH)	14.5 ± 0.3	24.8
N_2 (no microsomes 8.0 mM NADPH)	14.3 ± 0.3	24.4

[a]Values for dye disappearance are average ± SEM of triplicate incubations. Reactions were performed at 37°; 3 ml incubations contained 50 μM sulfonazo III, 100 μg protein/ml, 0.39 mM NADPH (except where change is noted), in buffer, pH 7.4, KCl-Tris (150 mM and 20 mM).

anion radical should be partially protonated at pH 7.4. The expected rapid exchange of this proton with the water protons will abolish this hyperfine interaction and preserve a single line at the center of the spectrum.

When sulfonazo III (1 mM) is incubated under an N_2 atmosphere with rat hepatic microsomes (1 mg/ml) an ESR spectrum of the sulfonazo III anion free radical appears at its maximum amplitude within five seconds of the addition of either NADPH or NADH (0.39 mM), and then decays after 10-15 seconds. The short rise time of the signal implies a rapid rate of radical decay. In subsequent studies a NADPH generating system was used in order to increase the time interval that the steady-state signal is at its maximum amplitude.

The reduction of the azo group to a hydrazo linkage destroys the conjugation of the aromatic rings causing the loss of the azo color. The reduction of bisazo dyes has been reported to proceed in a stepwise manner (17). We have not identified the products of sulfonazo III reduction in the microsomal

Table III. The Effects of Atmospheric Conditions and Heat Denaturation on the Steady-State ESR Signal of the Sulfonazo Anion Free Radical Metabolite in Rat Hepatic Microsomal Incubations

Atmosphere	Relative Amplitudes[a]
N_2	100.0[b] ± 0.1[c]
Air	7.3 ± 5.2
Air (after 2.5 min)	98.2 ± 1.0
CO	96.0 ± 0.9
N_2 (microsomes heated 57° for 15 min)	1.0 ± 0.2
N_2 (no microsomes)	0.0 ± 0.6
N_2 (no microsomes x 10 generating system)	0.5 ± 0.1

[a]The instrumental settings described in Fig. 1B with a gain of 1.0×10^3 were used to monitor the steady-state amplitude.

[b]Glucose-6-phosphate dehydrogenase (0.67 μ /ml) was added to a 37° incubation containing 1.0 mg protein/ml, 5 mM sulfonazo III, 0.87 mM NADP and 10.9 mM glucose-6-phosphate. A one and one-half minute interval was required for full activity apparently due to inhibition by NADP. Even with a NADPH generating system the steady-state signal decreased and after four minutes was 92% of the maximum value.

[c]The values reported are the mean of duplicate ± one-half the range. Sulfonazo III (5 mM) in buffer, pH 7.4, KCl-Tris-$MgCl_2$ (150 mM, 20 mM and 5 mM), had a signal of relative amplitude 1.9 from a trace of a stable free radical impurity. This signal was not present in buffer and was subtracted from all the relative amplitudes above.

incubations except to note the shift of the absorption maximum from 573 nm to 427 and 555 nm. These maxima may be due to the formation of the monoazo dye metabolite. In any case, this species is completely decolorized by sodium dithionite, but is resistant to microsomal azoreductase. With NADPH as the source of reducing equivalents, the rate of sulfonazo III (50 μM) disappearance under a nitrogen atmosphere results in the total consumption of the bisazo dye in less than one minute (Table II).

When the incubation is not deoxygenated, the ESR signal is only 7% of the nitrogen-purged incubations. Thereafter, the signal increases and at 2.5 minutes reaches the same amplitude as the deoxygenated samples (Table III).

Preliminary oxygen uptake data indicates that these incubations consume all of the oxygen by this time (18,19). In nonaqueous solvents the azo anion free radicals are oxidized by molecular oxygen to give the original azo compounds (20,21). In the presence of air the rate of dye disappearance is only 3% of that seen in anaerobic incubations (Table II). Microsomal azoreduction of neoprontosil has been reported to be completely inhibited by oxygen (7), although other azo reductases are often insensitive to oxygen (22-24). On the other hand, the effect of CO on both the ESR steady-state signal and the dye disappearance is minimal.

Heat denaturation causes an almost total loss of the ESR signal, and incubations without microsomes have no significant activity even when the NADPH generating system is increased ten-fold (Table II). In contrast to the radical metabolite signal, NADPH (8.0 mM) can nonenzymatically reduce the azo dye (Table II) (7). This dye disappearance is insensitive to oxygen and is apparently responsible for the residual activity of the heat denatured microsomes.

Discussion: These studies indicate that an azo anion free radical metabolite is formed enzymatically in microsomal incubations during the azo reduction of sulfonazo III. The primary microsomal azoreductases have been shown to be NADPH-cytochrome c reductase, although, NADH-cytochrome b_5 reductase, P-450, unbound flavins, and other pathways are also involved (7). This microsomal flavoenzyme also catalyzes the reduction of quinones to semiquinones (25,26) and nitroaromatic compounds to their anion radicals (1-3). The rate of reduction of azo compounds by unbound flavins (6,27,28) is proportional to the reduction potential of the azo compounds (29), as might be expected for a simple electron transfer reaction. For these reasons we propose that NADPH-cytochrome c reductase donates an electron to azo substrates via a shuttle between the fully-reduced flavin and the half-reduced flavin, as this flavoenzyme does during cytochrome c reduction (Figure 2). Azo anion free radicals are known to disproportionate in aqueous solutions to form the hydrazo

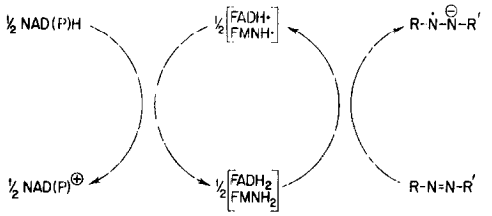


Fig. 2. The proposed mechanism of azo anion free radical formation. The exact scheme of electron donation via NADPH-cytochrome c reductase does not alter our conclusions (30,31).

intermediate (32), but at present we have no kinetic evidence that the azo anion free radical is an obligate metabolite of microsomal azo reduction.

The nonenzymatic anaerobic reduction of sulfonazo III by NADPH (Table II) would appear to be the result of a hydride transfer from NADPH to form the hydrazo intermediate, because the ESR signal of the azo anion radical cannot be detected in the absence of microsomes (Table III). In contrast to the microsomal azoreductase, this nonenzymatic azo reduction is oxygen insensitive (Table II). This result appears to exclude the possibility that the azo anion free radical forms by the comproportionation of the hydrazo intermediate with the azo substrate.

The formation of an oxygen sensitive azo anion free radical metabolite suggests that the O_2 inhibition of azoreductases could result from the rapid reoxidation of the azo anion radical by the sequence:

a) $R\text{-}N{=}N\text{-}R' \xrightarrow{e^-} [R\text{-}N\text{-}N\text{-}R']^{\cdot-}$

b) $[R\text{-}N\text{-}N\text{-}R']^{\cdot-} + O_2 \longrightarrow R\text{-}N{=}N\text{-}R' + \dot{O}_2^-$

In this mechanism, the rapid air oxidation of the azo anion free radical is proposed to occur by a one-electron transfer from the azo anion free radical to O_2. In such a scheme there would be no net reduction of the azo compound since the parent azo compound would be reformed. The azo dye would thereby catalyze the production of superoxide anion free radical from molecular oxygen. This

mechanism for the oxygen inhibition of microsomal azoreductase predicts that oxygen will be consumed and superoxide anion free radical will be produced in aerobic incubations containing the azo substrate, microsomes, and NADPH. Preliminary results confirm this expectation (18,19). Hence, the absence of dye disappearance or of the formation of hydrazo or amine reduction products under aerobic conditions does not imply that the azo anion free radical has not been formed. In particular, the absence of *in vivo* azo dye reduction in germ free rats (33) or rats treated with antibiotics (34) does not imply that the azo anion radical does not form in hepatic tissue, but may only be indicative of the relatively high oxygen tension in the liver compared with that of the intestines

Sulfonazo III is used in the titrimetric determination of sulfates and organic sulfur (35), and is structurally related to the monoazo food dyes FD & C Red 2 (amaranth) and FD & C Red 40. We have reported a very weak ESR signal from microsomal incubations of FD & C Red 2, which is similar to that seen in Figure 1B (18), but we have not as yet measured its g-value. The detection of the sulfonazo III anion free radical metabolite is probably facilitated by the unusually rapid rate of microsomal azo reduction seen with the sulfonazo III substrate.

DPPH, which is chemically related to the azo anion free radicals, is known to abstract hydrogen from tocopherol, cysteine (36) and amino acids (37). This reaction, as well as the azo anion free radical mediation of superoxide formation, may be involved in the toxicities of azo drugs and dyes.

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