Generation of Free Radical Metabolites and Superoxide Anion by the Calcium Indicators Arsenazo III, Antipyrylazo III, and Murexide in Rat Liver Microsomes*

(Received for publication, July 11, 1983)

Roberto Docampo, Silvia N. J. Moreno, and Ronald P. Mason
From the Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

At the concentrations usually employed as a Ca\(^{2+}\) indicator, arsenazo III undergoes a one-electron reduction by rat liver microsomes to produce an azo anion radical as demonstrated by electron spin resonance spectroscopy. Either NADH or NADPH can serve as a source of reducing equivalents for the production of this free radical by rat liver microsomes. The steady state concentration of the azo anion radical is proportional to the square root of the protein concentration, suggesting that the radical decays through a nonenzymatic second order process. The steady state concentration of the azo anion radical is not altered in the presence of metyrapone or CO, and is decreased in the presence of NADP* or \(p\)-hydroxymercuribenzoate. These observations suggest that the formation of arsenazo III anion radical is mediated through NADPH-cytochrome P-450 reductase and not by cytochrome P-450. Under aerobic conditions, addition of arsenazo III to rat liver microsomes produces an increase in electron flow from NAD(P)H to molecular oxygen, generating both superoxide anion and hydrogen peroxide. The steady state concentration of the azo anion radical, but neither oxygen consumption nor superoxide anion formation, is enhanced by calcium and magnesium, suggesting an enhanced azo anion radical stabilization by complexation with the metal ions. Accordingly, the arsenazo III anion radical signal is abolished in the presence of paramagnetic metal ions (Fe\(^{3+}\), Ga\(^{3+}\), and Ni\(^{2+}\)) and enhanced in the presence of other diamagnetic metal ions (La\(^{3+}\)). Antipyrylazo III is less effective than arsenazo III in increasing superoxide anion formation by rat liver microsomes, and gives a much weaker ESR spectrum of an azo anion radical. Murexide is reduced to the monodehydro-5,5'-iminobarbitaluric acid radical by rat liver microsomes, and its efficiency as a superoxide anion generator is intermediate between arsenazo III and antipyrylazo III.

Metallochromic indicators are substances that undergo color changes when the concentration of free metal ion in the solution changes (1). This is the result of the formation of a complex between the indicator and the metal ion with consequent changes in the electronic energy levels of the indicator and the wavelength at which the indicator absorbs light (1).

The three most commonly used indicators of Ca\(^{2+}\) transport in biological systems are ammonium purpurate (murexide), 2,2'-[1-(1,8-dihydroxy-3,6-disulphonaphthalene-2,7-bisazo)bis-(benzenearsonic acid)] (arsenazo III), and bis(4-antipyrylazo)-4,5-dihydroxy-2,7-naphthalenedisulfonic acid (antipyrylazo III). Murexide has been recognized for a long time as a Ca\(^{2+}\) indicator (2) and has been applied to the measurements of Ca\(^{2+}\) transport in isolated sarcoplasmic reticulum (3), mitochondria (4), and cell suspensions (5). Arsenazo III and antipyrylazo III have only recently been recognized as Ca\(^{2+}\) indicators (6, 7) and have been applied to the measurements of Ca\(^{2+}\) transport in isolated mitochondria (8–10), sarcoplasmic reticulum (7, 11, 12), liver microsomes (13), chromaffin vesicles (14), and also for measurements of ionized Ca\(^{2+}\) within single cells (15–17).

Recent studies have shown that sulfonazo III, another diazonaphthol dye closely related to arsenazo III and antipyrylazo III, is rapidly reduced by rat liver microsomes to an azo anion radical whose autoxidation generates superoxide anion (18, 19). In addition, reduction of murexide by cysteine has been shown to produce a free radical as demonstrated by ESR spectroscopy (20).

Due to the importance of metallochromic indicators as powerful tools in the study of Ca\(^{2+}\) transport in biological systems, we investigated the possibility of their reduction by rat liver microsomes.

**MATERIALS AND METHODS**

CD male rats (150-200 g, Charles River, Inc.) were used in the experiments. They were fed standard rat chow and water ad libitum and were not fasted prior to use. The animals were killed and their livers were rapidly removed and processed. Microsomes were prepared as described previously (21) and kept on ice until use.

**ESR observations were made at room temperature, 24 °C, with a Varian E-109 spectrometer equipped with a TMS110 cavity or a Varian E-104 spectrometer as described previously (23, 24). Spin trapping experiments were carried out as described before (25) using 100 mM DMPO\(^\cdot\) purified according to Buettner and Oberley (26).

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated as "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Career Investigator, Consejo Nacional de Investigaciones Científicas y Técnicas. Permanent address, Centro de Investigaciones Bioenergeticas, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina. To whom all correspondence should be addressed at Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, P. O. Box 12235, Research Triangle Park, NC 27709.

§ Visiting Fellow, National Institutes of Health.
Oxygen uptake was determined with a Clark electrode (YSI-5351, Yellow Springs Instrument Co.) in a water-jacketed glass vessel at 30 °C.

Optical spectra of arsenazo III/microsome incubations were recorded on an Aminco Model DW-2A spectrophotometer in the split beam mode at 30 °C. The protein concentration was determined as described previously (25).

RESULTS

Fig. 1 shows that oxygen consumption was increased over 4-fold when arsenazo III was introduced into a reaction mixture containing rat liver microsomes and NADPH. The arsenazo III-induced oxygen uptake depended on all the components of the system, the reaction rate being negligible in the absence of either NADPH or microsomes or after the thermal denaturation of the microsomes (not shown). The accumulation of H₂O₂ in the medium was indicated by the increase in oxygen concentration after catalase addition and a subsequent decrease in the oxygen consumption, indicating that the oxygen consumed was partially transformed to H₂O₂. The addition of superoxide dismutase after catalase significantly decreased the stimulation of oxygen uptake, presumably by increasing the rate of disproportionation of the superoxide anion formed by autoxidation of arsenazo III (19), and/or by abolishing the inhibition of catalase by O₂⁻ (27):

\[
\text{O}_{2}^{-} + \text{O}_{2}^{-} \rightarrow \text{H}_{2}\text{O}_{2}
\]

Fig. 2A shows that NADH was less effective than NADPH as an electron donor for arsenazo III reduction. The concentration-effect curve for arsenazo III is also shown in Fig. 2B. It is observed that stimulation of oxygen utilization was in accordance with saturation kinetics (Kₐ = 35 μM; V = 12.5 nmol/min/mg of protein in the presence of NADPH; Kₐ = 87 μM; V = 4.16 nmol/min/mg of protein in the presence of NADH). In contrast to these results, antipyrylazo III and murexide stimulated oxygen consumption at much higher substrate concentrations than arsenazo III (Fig. 3). This stimulation was more significant in the presence of NADPH as electron donor (Kₐ = 10 mM; V = 10 nmol/min/mg of protein for antipyrylazo III; Kₐ = 2 mM; V = 10 nmol/min/mg of protein for murexide).

When arsenazo III (50-100 μM) was incubated under a nitrogen atmosphere with rat liver microsomes in the presence of NADPH, an unresolved single line ESR spectrum similar to that observed with sulfonazo III incubations (18, 19) was detected (Fig. 4A). The radical formation depended on all three components: rat liver microsomes, NADPH, and arsenazo III. Omission of NADPH or the heating of the microsomes in a steam bath for 10 min led to a total loss of activity. The NADPH alone did not produce observable concentrations of the arsenazo III radical. Although NADPH could replace NADH in these incubations, its activity was about 50% lower (Fig. 4B). The spectra of the anion radical could not be observed under aerobic conditions; however, the signal appeared when the dissolved oxygen in the incubation was consumed. Following this lag period a steady state concentration was achieved. The steady state concentration was proportional to the square root of the microsomal protein concentration (Fig. 5), indicating that radical decay was kinetically second order. This result implies nonenzymatic disproporportionation (or dimerization) of the radical (28). No antipyrylazo III or murexide radical could be discerned under the same conditions. However, at the same protein concentration, a weak signal was evident at 2 mM antipyrylazo III (Fig. 6A),
and a well resolved signal of the monodehydro-5,5′-iminobarbituric acid (20) was observed at 1 mM murexide (Fig. 6B). These signals also depended on all the components of the system and were not observable under aerobic conditions.

Aerobic incubations containing arsenazo III and the spin trap DMPO resulted in an ESR spectrum containing contributions from both the superoxide radical adduct and the hydroxyl radical adduct (Fig. 4C). The assignment of these adducts were made on the known hyperfine couplings (29). In support of this assignment, superoxide dismutase (10 µg/ml) completely inhibited the formation of both spin adducts (Fig. 4E). Catalase (250 µg/ml) had no effect on either of the observed spectra (not shown). Both of these results show that the hydroxyl spin adduct did not arise from hydrogen peroxide via either a Fenton, a Haber-Weiss, or some other H2O2-dependent reaction (30). Thus, the DMPO hydroxyl radical adduct probably was formed by the decomposition of the DMPO superoxide adduct (30) and/or by the trapping of the hydroxyl radical generated by the decomposition of the DMPO superoxide radical adduct (31). Under the experimental conditions used, the spectra of the DMPO adducts were not observed in the absence of NADPH or rat liver microsomes (not shown), and very weak spectra of the superoxide dismutase-sensitive DMPO adducts were observed in the absence of arsenazo III (Fig. 4D), attributable to the NADPH-supported superoxide anion formation by liver microsomes (32). In the presence of high concentrations of antipyrylazo III (2 mM) or murexide (1 mM), weak superoxide dismutase-sensitive DMPO adducts hardly exceeding the basal signals could be observed (approximately 25 and 15% of the signal amplitude of Fig. 4C, respectively).

The effects of known inhibitors of NADPH-cytochrome P-450 (c) reductase, DT-diaphorase, and cytochrome P-450 activity on arsenazo III anion radical formation by rat liver microsomes are shown in Table I. Dicumarol did not decrease the steady state concentration of the radical. The lack of dicumarol inhibition rules out a major role for microsomal DT-diaphorase (33) as the azoreductase. The inhibition produced by NADP+, a competitive inhibitor of NADPH-cytochrome P-450 (c) reductase, is consistent with this flavoprotein being the arsenazo III reductase. The same conclusion is supported by the effect of p-hydroxymercuibenzoate, an inhibitor of NADPH-dependent electron transport in liver microsomes (33-35). The lack of inhibition of arsenazo III anion radical formation by CO and metyrapone, two highly specific inhibitors of the microsomal electron transport system at the level of cytochrome P-450 (36), confirms that this cytochrome is not involved in azo anion radical formation from arsenazo III. In agreement with these results, metyrapone did not inhibit arsenazo III-stimulated oxygen consumption in the presence of NADPH (arsenazo III-stimulated oxygen consumption was 6.0 ± 0.5 nmol of O2/min/mg in the absence and 6.0 ± 0.6 nmol of O2/min/mg in the presence of 1 mM metyrapone).
In order to evaluate the effect of Ca$^{2+}$ and Mg$^{2+}$ on the free radical formation induced by arsenazo III, the azo anion radical formation was measured with or without the addition of Ca$^{2+}$ or Mg$^{2+}$. Even without the addition of these cations, a weak azo radical signal approximately 4 times lower than that in Fig. 4A was still present (Fig. 7). The signal intensity increased dramatically after addition of Ca$^{2+}$ to the incubation medium and reached a plateau at approximately 100 $\mu$M Ca$^{2+}$ (Fig. 7). Addition of Mg$^{2+}$ to the incubation medium also increased the azo anion radical intensity (Fig. 7), reaching a plateau at approximately 300 $\mu$M Mg$^{2+}$. In contrast, the arsenazo III-stimulated oxygen consumption and the DMOPO hydroxyl adduct signals obtained in the presence of arsenazo III were not significantly increased by increasing Ca$^{2+}$ or Mg$^{2+}$ concentrations up to 400 $\mu$M (Table III). As this enhanced free radical steady state concentration by cations may be attributed to azo anion radical-stabilization by complexation with the metal cations, the effect of different diamagnetic and paramagnetic metal ions was investigated (Table II). As expected, the arsenazo III anion radical signal was abolished in the presence of paramagnetic metal ions (Fe$^{3+}$, Gd$^{3+}$, and Ni$^{2+}$). A key experiment was the comparison of effects of La$^{3+}$ (diamagnetic) and Gd$^{3+}$ (paramagnetic), which have similar chemical properties. At similar concentrations, addition of Gd$^{3+}$ eliminated the free radical signal, while a very significant increase was found for La$^{3+}$. Other diamagnetic metal ions such as Zn$^{2+}$, Cd$^{2+}$, Bi$^{3+}$, and Pb$^{2+}$ did not increase the arsenazo III anion radical signal significantly, possibly indicating the lack of azo anion radical-stabilization.

**Table I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>CO</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>Metyrapone (1.0 mM)</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>NADP$^+$ (1 mM)</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>NADP$^+$ (3 mM)</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate (0.05 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Dicumarol (0.1 mM)</td>
<td>102 ± 6</td>
</tr>
</tbody>
</table>

**Fig. 5.** The relative steady state concentrations of the arsenazo III anion free radical and the square of the relative steady state concentrations versus the protein concentration of rat liver microsomes.

**Fig. 6.** ESR spectra of rat liver microsomal incubations in the presence of antipyrylazo III and murexide. A, spectrum obtained from an incubation of rat liver microsomes (1.3 mg/ml) containing 2 mM antipyrylazo III, 1 mM NADPH in 150 mM KCl, 50 mM Tris, and 5 mM MgCl$_2$ (pH 7.4). The nominal microwave power was 20 milliwatts, and the modulation amplitude was 6.7 G. The scan time was 2 min, and the time constant was 2 s. B, identical with A, but with 1 mM murexide instead of antipyrylazo III. The hyperfine coupling constants were $\alpha_H = 8.08$ G and $\alpha_N = 9.08$ G. The nominal microwave power was 20 milliwatts, the modulation amplitude was 0.33 G, the scan time was 16 min, and the time constant was 1 s.

**Fig. 7.** Effect of different concentrations of Ca$^{2+}$ and Mg$^{2+}$ on the steady state ESR signal of arsenazo III anion radical. The same ESR cell remained in the cavity throughout the experiment to minimize any artifact due to differences in cell position. To maximize the signal-to-noise ratio, the instrument settings were 20 milliwatts of microwave power and 6.7 G of modulation amplitude. The values are the average ± S.D. of three incubations. Other conditions are as in Fig 4A and "Materials and Methods."
The same ESR cell remained in the cavity throughout the experiment to minimize any artifact due to differences in cell position. The instrument settings were 20 milliwatts of microwave power and 8.7 G of modulation amplitude. The values are the averages ± S.D. The number of determinations are in parentheses. The incubations contained 1.25 mg/ml of microsomal protein, 100 μM arsenazo III, and 1 mM NADPH in 100 mM Tris buffer (pH 7.4) and 0.1 mM of each salt or chelating agent. Other conditions are as in Fig. 4A and under "Materials and Methods."

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative amplitude</th>
<th>Linewidth</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 10</td>
<td>13.3 ± 0.2 (10)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>320 ± 15</td>
<td>11.0 ± 0.3 (7)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>200 ± 12</td>
<td>12.7 ± 0.2 (9)</td>
</tr>
<tr>
<td>LaCl₃</td>
<td>1000 ± 20</td>
<td>10.1 ± 0.5 (9)</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>90 ± 8</td>
<td>12.5 ± 0.6 (5)</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>40 ± 6</td>
<td>11.4 ± 0.8 (4)</td>
</tr>
<tr>
<td>PbCl₂</td>
<td>60 ± 4</td>
<td>10.4 ± 0.4 (6)</td>
</tr>
<tr>
<td>BiCl₃</td>
<td>30 ± 4</td>
<td>10.7 ± 0.8 (4)</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>GdCl₃</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>DTPA</td>
<td>140 ± 10</td>
<td>14.4 ± 0.2 (7)</td>
</tr>
<tr>
<td>EGTA</td>
<td>290 ± 15</td>
<td>14.3 ± 0.2 (9)</td>
</tr>
<tr>
<td>EDTA</td>
<td>110 ± 12</td>
<td>13.9 ± 0.1 (4)</td>
</tr>
</tbody>
</table>

* The reaction mixture contained microsomes (2 mg/ml), 100 μM arsenazo III, and 1 mM NADPH, in the same buffer as in Table II. The values are nanomoles of O₂/min/mg of protein.

** The incubation contained microsomes (1.5 mg/ml), 100 μM arsenazo III, 1 mM NADPH, and 100 mM DMPO in the same buffer as in Table II. Other conditions are as in Fig. 4C. The same ESR cell remained in the cavity throughout the experiment.

** The values are the averages ± S.D. of at least three determinations.

** ND, not determined.

* The reaction mixture contained microsomes (2 mg/ml), 100 μM arsenazo III, and 1 mM NADPH, in the same buffer as in Table II. The values are nanomoles of O₂/min/mg of protein.

** The incubation contained microsomes (1.5 mg/ml), 100 μM arsenazo III, 1 mM NADPH, and 100 mM DMPO in the same buffer as in Table II. Other conditions are as in Fig. 4C. The same ESR cell remained in the cavity throughout the experiment.

** The values are the averages ± S.D. of at least three determinations.

** ND, not determined.

by these cations. It is known that certain chelating agents can improve the use of arsenazo III as a metallochromic indicator by complexing interfering metal ions (37). Accordingly, EGTA and DTPA, probably by chelating contaminating paramagnetic ions in the incubation medium, also increased the arsenazo III anion radical signal. Concomitant with the large changes in signal amplitude there were significant changes in the linewidth which varied with the metal ions. Broad spectra appeared in the presence of Ca⁺², La⁺³, Zn⁺², Pb⁺², and Bi⁺³. The linewidth changes were much too small to account for, and do not correlate with, changes in the signal amplitude. As occurred with Ca⁺² and Mg⁺², arsenazo III-stimulated oxygen consumption in the presence of other metal ions and chelators did not correlate with changes in signal amplitude of the azo anion radical (Table III). Arsenazo III-stimulated oxygen consumption was not significantly increased by 100 μM DTPA, EGTA, EDTA, or FeCl₂ and was only slightly increased by 100 μM ZnCl₂, PbCl₂, BiCl₃, and GdCl₃. Interestingly, CdCl₂ and LaCl₃ increased and NiCl₂ decreased significantly arsenazo III-stimulated oxygen consumption by rat liver microsomes.

The arsenazo III anion free radical could also be observed by visible spectroscopy (Fig. 8). Visible spectroscopy showed the decrease in optical density at 575 nm due to reduction of the azo dye and a transient azo anion radical absorbance at 700 nm (Figs. 8, A and B). This absorbance at 700 nm could not be observed under aerobic conditions; however, the absorbance at 700 nm appeared when the dissolved oxygen in the incubation was consumed, reached a maximum that remained at steady state for 2 min, and then decayed. That steady state concentration was also proportional to the square root of the protein concentration and increased on addition of Ca⁺² or Mg⁺² to the incubation mixture (not shown). This correspondence with the changes of the ESR spectra was the best evidences that the transient species at 700 nm was, in fact, the azo anion radical.

**DISCUSSION**

The observations described here indicate that arsenazo III is reduced by rat liver microsomes to an azo anion radical,
the reoxidation of which is accompanied by the formation of active oxygen radicals. In fact, arsenazo III (a) induced additional microsomal oxygen utilization (Figs. 1 and 2); (b) increased the rate of O₂ formation (Fig. 1C); and (c) increased H₂O₂ production by microsomes (Fig. 1). In addition to these effects, evidence for the arsenazo III anion radical formation was obtained by electron spin resonance spectroscopy (Figs. 4, A and B, 5, and 7) and visible spectroscopy (Fig. 8). The effect of inhibitors in Table I indicates that the initial reduction of the drug was initiated by NADPH-cytochrome P-450 (e) reductase. The lack of effect of specific inhibitors of cytochrome P-450 (metyrapone and CO) on arsenazo III anion radical formation indicates that cytochrome P-450 is not involved in the initial reduction step.

Free radical formation has been postulated as a mechanism of drug-induced injury for a wide range of chemicals including anticancer and antiparasitic compounds (38, 39). Generally, the damage which results is attributed to either the reactivity of the drug radical or the subsequent production of oxygen radicals. Interestingly, arsenazo III is reportedly nontoxic when loaded into cells at concentrations up to 1 mM (1, 40), although prolonged incubation of muscle cells for several days at concentrations in excess of 1 mM inhibited growth and at 5 mM caused cell death (41). The trypanocidal effect of only high concentrations of arsenazo III against Trypanosoma brucei has also been reported (42), and one may speculate that arsenazo III-induced redox cycling cannot overwhelm the cellular defenses against oxygen radicals in the cells in which it is injected (1, 39) or, like trypan blue, does not penetrate intact cells (41, 42). However, the ability of arsenazo III to induce superoxide anion and hydrogen peroxide formation at the concentrations usually employed as an indicator may represent a serious disadvantage in some in vitro experiments.

In the presence of rat liver microsomes, Ca²⁺ increases the azo anion radical steady state concentration, and Mg²⁺ can replace Ca²⁺. This enhanced free radical steady state concentration by cations may be due to radical-stabilization by complexation with the metal ions as occurs with o-semiquinones (43, 44). The complex may involve the azo and the hydroxyl group, and superoxide anion formation by rat liver microsomes at a concentration one order of magnitude higher than that of arsenazo III. Furthermore, the results obtained allow one to assume that the effect of antipyrilazo III on oxygen radical formation in intact cells must necessarily be small, hardly exceeding the basal levels. In addition, murexide was less effective than arsenazo III, although more effective than antipyrilazo III, in increasing oxygen consumption and superoxide anion formation by rat liver microsomes. The difference noted among these three indicators’ capability to generate free radicals may be significant in the election of the most suitable reagent for a particular experiment.

REFERENCES
Generation of free radical metabolites and superoxide anion by the calcium indicators arsenazo III, antipyrylazo III, and murexide in rat liver microsomes.
R Docampo, S N Moreno and R P Mason


Access the most updated version of this article at [http://www.jbc.org/content/258/24/14920](http://www.jbc.org/content/258/24/14920)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/258/24/14920.full.html#ref-list-1](http://www.jbc.org/content/258/24/14920.full.html#ref-list-1)