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FREE RADICAL METABOLISM OF HYDRALAZINE

BINDING AND DEGRADATION OF NUCLEIC ACIDS

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Abstract—The binding of hydralazine, a hydrazine-containing hypotensive drug, to nucleic acids has been studied. Binding of this drug to biopolymers was assayed using spin-trapping techniques in the presence of various metal ions, which produce free radical intermediates from hydralazine [B. K. Sinha and A. G. Motten, *Biochem. biophys. Res. Commun.* **105,** 1044 (1982)]. Some interaction was detected with the single-stranded nucleic acids. Hydralazine binds strongly to the native DNA, most likely by intercalation of the drug into DNA bases. In the presence of nucleic acids and metal ions, hydralazine stimulated the production of $OH^{\cdot}$ radicals which was inversely proportional to the degree of binding. Aldehyde formation in DNA was also induced by hydralazine which was stimulated by superoxide dismutase and inhibited by catalase.

Derivatives of hydrazine occur naturally in tobacco and mushrooms [1, 2]. Hydrazines and their derivatives are used in industry, agriculture and medicine. Unfortunately, these compounds are extremely toxic and cause irreversible cellular damage [3, 4]. Furthermore, a large number of hydrazines have been shown to be mutagenic and carcinogenic [5, 6]. Hydralazine (1-hydrazinophthalazine), an aromatic hydrazine derivative, is used in the management of chronic hypertensive disease [7], but treatment with hydralazine over a long period has been associated with induction of systemic lupus erythematosus (SLE) [8]. Recently, Shaw *et al.* [9] and Williams *et al.* [10] have shown that hydralazine induces a base pair substitution mutation in bacterial systems. Additionally, Toth [11] has reported an increased incidence of lung tumor in mice exposed to hydralazine. In limited studies, hydralazine has been shown to bind to DNA and react with thymidine and deoxycytidine bases [12, 13]. Such binding of hydralazine to DNA may be important in its biochemical properties; however, neither the etiology of SLE induction nor the molecular mechanism of hydralazine carcinogenicity is known at this time.

Misra and Fridovich [14] have suggested that free radical intermediates are formed during metal ion-catalyzed oxidation of phenylhydrazine. Recent studies by Hill and Thornalley [15] and Augusto *et al.* [16] have provided evidence for the generation of carbon-centered free radicals from both phenylhydrazine and ethylhydrazine. Furthermore, we have shown that hydralazine produces nitrogen-centered radicals during oxidation, catalyzed by both metal ions and enzymes [17, 18]. Free radical intermediates and the resultant oxygen radicals ($O_2^{\cdot -}$, $OH^{\cdot}/H_2O_2$) have been implicated in mutagenesis and carcinogenesis [19, 20] and are known to augment DNA strand breaks [21]. The purpose of the present work was 2-fold: (a) to study the binding of hydralazine to nucleic acids and (b) to elucidate the role of $O_2^{\cdot -}/OH^{\cdot}$ radicals produced from hydralazine in DNA degradation. Studies presented here may be useful in understanding the molecular mechanism of hydralazine toxicity.

MATERIALS AMD METHODS

Hydralazine·HCl, xanthine oxidase (EC 1.2.3.2), hypoxanthine, superoxide dismutase (SOD), calf thymus DNA (highly polymerized, type I), and yeast RNA (type XI) were obtained from the Sigma Chemical Co. (St. Louis, MO). Diethylenetriaminepentaacetic acid (DETAPAC) and the spin trap 5,5-dimethyl-1-pyrroline oxide (DMPO) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). DMPO was purified by two distillations before use. Catalase was purchased from the Boehringer-Mannheim Co. (Indianapolis, IN), and Chelex-100 was obtained from the Bio-Rad Co. Laboratories (Richmond, CA).

Binding studies of hydralazine with nucleic acids were carried out in metal ion free buffers which were prepared by dissolving sodium phosphate or sodium borate in glass double-distilled water, adjusting the pH, and then filtering through a Chelex-100 column (20–40 g/l). The metal ions present in the nucleic acids were removed by dialyzing DNA or RNA solutions against 0.1% DETAPAC (18 hr, at 4°) followed by extensive dialysis against the desired metal ion free buffers (48 hr, at 4°). Denatured DNA was prepared by heating to 100° for 20 min, rapidly quenching in ice, and then repeating the procedure.

The metal ion catalyzed oxidation was carried out with Cu^{2+} ($CuCl_2$), Fe^{2+} ($FeSO_4$), and Fe^{3+} ($FeCl_3$)

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in the presence of nucleic acids at pH 7.4 (10 mM phosphate–100 mM NaCl) or pH 10.0 (50 mM borate buffer). A typical reaction mixture contained 2 mM hydralazine, 100 mM DMPO, 3 mg/ml nucleic acids (1×10^{-2} M in nucleotides) and 50 μM metal ion. Superoxide mediated oxidation of hydralazine was carried out by incubating 2 mM hydralazine with 50 μM hypoxanthine, and 0.065 units/ml xanthine oxidase in the presence of 100 mM DMPO and 1 mM DETAPAC at pH 10.0. The electron spin resonance spectra were recorded on a Varian E104 equipped with a TM_{238} cavity and a rapid sampler (Gilford, model 2443-AO) at room temperature (~22°).

The formation of thiobarbituric acid reactive compounds from deoxyribose cleavage of DNA was measured by thiobarbituric acid assay according to a published method [22]. Under our experimental conditions, the buffer (10 mM phosphate–10 mM NaCl), the drug, and the DNA did not interfere with the assay.

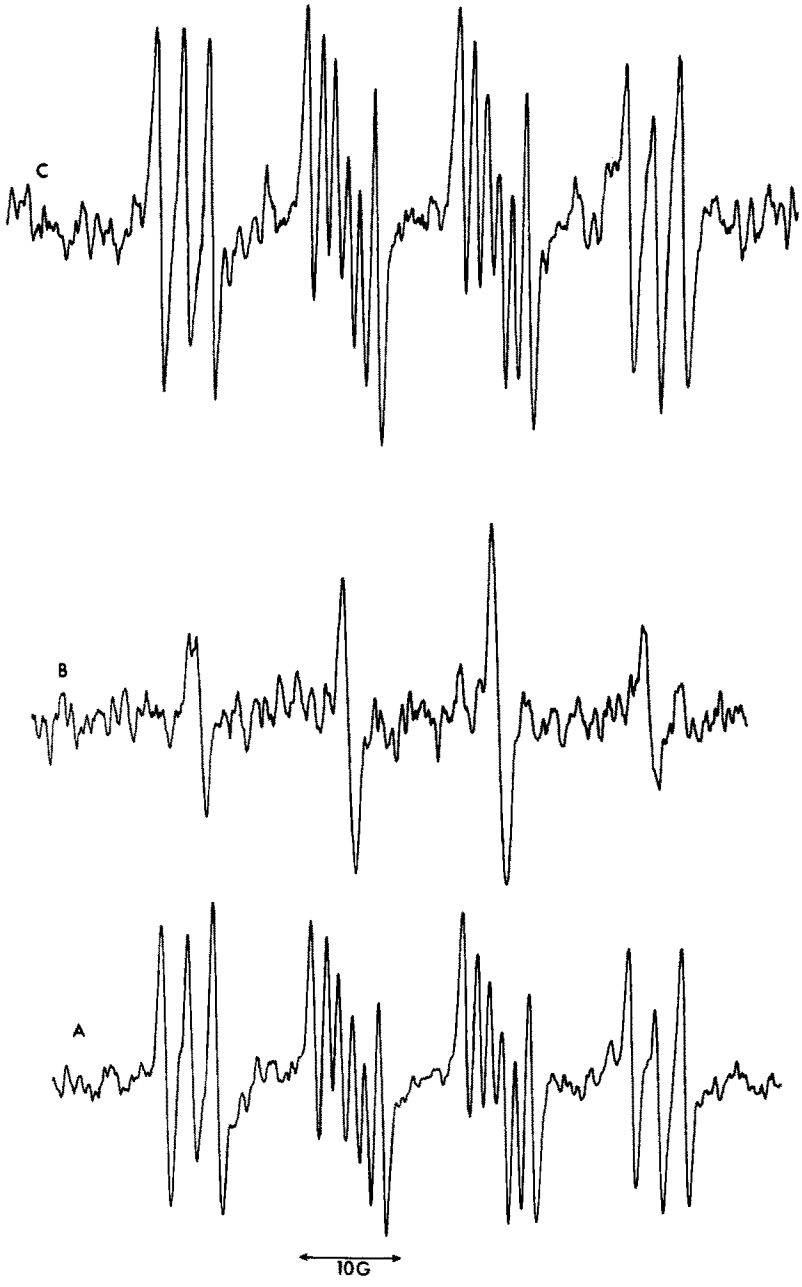

Fig. 1. Electron spin resonance (ESR) spectrum obtained from 2 mM hydralazine during (A) incubation with 0.065 units/ml xanthine oxidase, 50 μM hypoxanthine and 100 mM DMPO in the presence of 1 mM DETAPAC in borate buffer, pH 10.0, with $a^N = 15.0$ G, $a_\beta^H = 16.7$ G and $a_\beta^N = 2.5$ G. (B) Same as (A) except that it contained 10 μg/ml SOD and (C) same as (A) except that it contained 20 μg/ml catalase. ESR settings: field, 3360 G; field scan, 100 G; modulation frequency, 100 (k)H_z; modulation amplitude, 0.33 G; and microwave power, 20 mW. The receiver gain was 5×10^4 for (A), 1×10^5 for (B), and 1×10^5 for (C).

Table 1. Effects of nucleic acids (3 mg/ml) on the intensity of ESR spectrum of the nitrogen-centered DMPO adduct (low field line) during metal ion catalyzed oxidation of hydralazine (2 mM) in borate buffer at pH 10.0

Conditions*	Relative intensity Fe^{3+}	Fe^{2+}
Hydralazine	100	100
Hydralazine + DNA	49	38
Hydralazine + denatured DNA	74	70
Hydralazine + RNA	59	58

* The nucleic acids were incubated with hydralazine and DMPO for 10 min and then 50 μM metal ions were added. The spectrum was recorded at 10 min. The microwave power was 20 mW, the modulation amplitude was 0.33 G, and the receiver gain was 1×10^5.

RESULTS

Formation of nitrogen centered free radical by xanthine oxidase. Our previous studies on the metal ion catalyzed oxidative metabolism of hydralazine had implicated the intermediacy of $O_2^{\cdot-}$ in radical formation and propagation [17]. To prove that radical formation was $O_2^{\cdot-}$ dependent, hydralazine was incubated with xanthine oxidase, which is known to produce $O_2^{\cdot-}$ in the presence of hypoxanthine [23]. The incubation of the drug with either hypoxanthine or xanthine oxidase under aerobic conditions produced no detectable free radical intermediates. However, when both hypoxanthine and xanthine oxidase were present, a DMPO–radical adduct consisting of 18 lines with $a^N = 15.0$ G, $a_\beta^H = 16.7$ G and $a_\beta^N = 2.5$ G (Fig. 1) was detected. The 18-line spectrum is due to trapping of hydralazyl radical (RNHNH$^{\cdot}$) by DMPO [17]. The presence or absence of DETAPAC had little effect, suggesting that metal contaminants were not responsible for the radical generation from hydralazine. Furthermore, when the reaction was initiated under anaerobic conditions or in the presence of 10 μg/ml SOD, no radical could be detected. The presence of catalase in the incubation mixtures decreased the radical concentration by 20–30%. These observations suggest that $O_2^{\cdot-}$ is involved in radical formation from hydralazine.

Effects of nucleic acids on the formation of hydralazyl radical. If hydralazine binds to nucleic acids, the metal ion catalyzed formation of the radical intermediate would be expected to decrease in proportion to the degree of binding to the respective nucleic acids. The data in Table 1 demonstrate that the incubation of hydralazine with calf thymus DNA (P:D:5:1) at pH 10.0 in the presence of metal ions and DMPO decreased radical concentration by 40–50%. This would indicate that 40–50% of the drug or its metabolite is bound to DNA. In contrast, when heat-denatured DNA was used, 75% of hydralazine was free and available for the reaction with metal ions. Similarly, in the presence of RNA, about 60% of the drug was free.

Stimulation of OH$^{\cdot}$ radical formation. Hydralazine in the presence of nucleic acids and metal ions also stimulated the formation of OH$^{\cdot}$ radicals which was proportional to both the degree of binding and time (Fig. 2). The data show that OH$^{\cdot}$ radical production was inversely proportional to the binding of hydralazine to the nucleic acids. Similar results were obtained when Fe^{2+} was used and are shown in Fig.

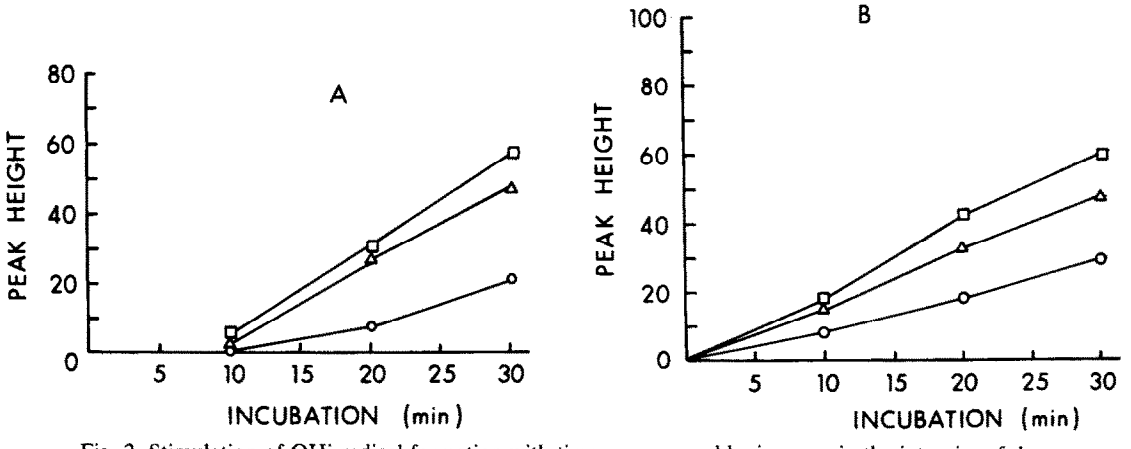

Fig. 2. Stimulation of OH$^{\cdot}$ radical formation with time as measured by increase in the intensity of the DMPO–OH adduct signal. (A) The incubation mixture contained 2 mM hydralazine, 100 mM DMPO, 50 μM Fe^{3+} and nucleic acids (3 mg/ml) at pH 10.0. Key: (○—○) DNA; (△—△) denatured DNA; and (□—□) RNA. (B) Same as (A) except that it contained 50 μM Fe^{2+}. Spectrometer settings were identical to Fig. 1 except that the receiver gain was 1×10^5.

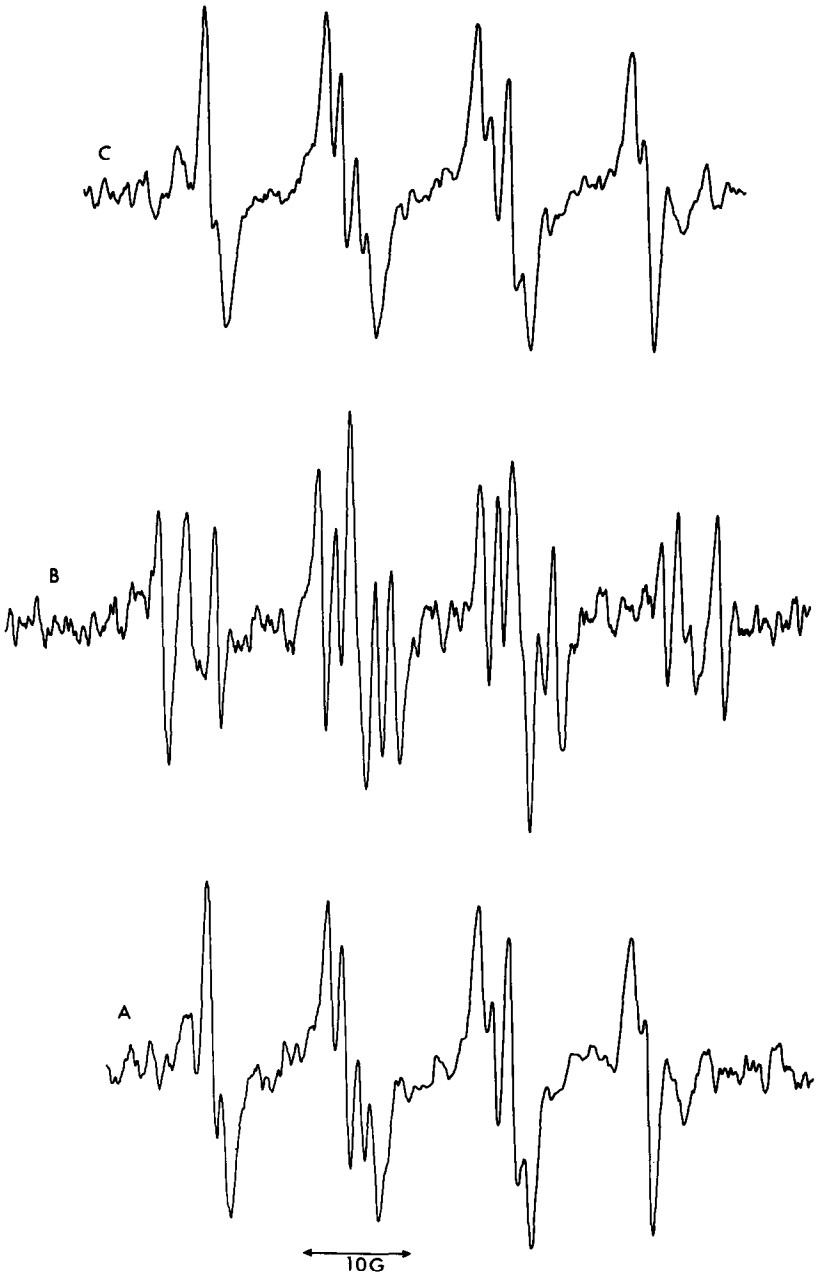

Fig. 3. ESR spectrum obtained from 2 mM hydralazine in the presence of (A) 50 μM Cu^{2+} and 100 mM DMPO at pH 7.4. (B) is identical to (A) except that it contained 10 μg/ml SOD, and (C) is identical to (A) except that it contained 20 μg/ml catalase. ESR settings: microwave power, 20 mW; modulation amplitude, 0.33 G; and the receiver gain 6.3×10^4 for (A), 1×10^5 for (B) and 5×10^4 for (C).

2B. Incubation of the nucleic acids with metals in the absence of hydralazine did not produce any $OH^{\cdot}$ radicals.

When hydralazine was incubated with Fe^{3+} at pH 7.4, only $OH^{\cdot}$ radicals were trapped by DMPO. Substitution of Cu^{2+} for Fe^{3+} at pH 7.4 resulted in the formation of a DMPO adduct with the following splitting constants: $a^N = 14.3$ G, $a_\beta^H = 11.7$ G; $a_\gamma^H = 1.2$ G (Fig. 3). These splittings are characteristic of a DMPO–OOH adduct [24]. No other adduct was detected. Addition of 10 μg/ml SOD inhibited the formation of the DMPO–OOH adduct, which was replaced by the 18-line nitrogen-centered DMPO adduct previously obtained. In addition, some DMPO–OH adduct was also present. The presence of DNA in the incubation mixture inhibited the formation of the DMPO–OOH adduct and only traces of the $OH^{\cdot}$ adduct could be detected. In contrast, RNA had no effect and the DMPO–OOH was rapidly formed.

Formation of aldehydes from DNA. Since hydralazine in the presence of nucleic acids and metal ions stimulated the formation of hydroxyl radicals, it is likely that this may induce aldehyde formation in DNA. The results presented in Table 2 show that addition of Fe^{2+}, in the absence of the drug, produced

Table 2. Formation of thiobarbituric acid reactive substances from calf thymus DNA in the presence of hydralazine (HZ) and metal ions and effects of superoxide dismutase (20 μg/ml), catalase (20 μg/ml) and EDTA (1 mM)*

Conditions	Additions	O.D.$_{532}$/mg DNA		
		10 min	20 min	30 min
DNA	Fe^{2+}	0.084 ± 0.002	0.116 ± 0.008	0.132 ± 0.001
DNA + HZ		0.052 ± 0.004	0.076 ± 0.007	0.096 ± 0.004
DNA + HZ	Fe^{2+}	0.096 ± 0.004	0.156 ± 0.016	0.140 ± 0.002
DNA + HZ + EDTA	Fe^{2+}	0.132 ± 0.001	0.216 ± 0.008	0.168 ± 0.008
DNA + SOD	Fe^{2+}	0.150 ± 0.008		
DNA + HZ + SOD	Fe^{2+}	0.270 ± 0.028		
DNA + HZ + catalase	Fe^{2+}	0.090 ± 0.008		
DNA	Cu^{2+}	0.256 ± 0.02	0.056 ± 0.01	0.312 ± 0.012
DNA + HZ	Cu^{2+}	0.304 ± 0.01	0.328 ± 0.012	0.360 ± 0.024
DNA + HZ + EDTA	Cu^{2+}	0.296 ± 0.004	0.328 ± 0.016	0.316 ± 0.012
DNA + SOD	Cu^{2+}	0.392 ± 0.01		
DNA + HZ + SOD	Cu^{2+}	0.568 ± 0.01		
DNA + HZ + catalase	Cu^{2+}	0.250 ± 0.01		

* Values are averages of duplicate incubations. Hydralazine (1 mM) was incubated with DNA (1.43×10^{-3} M nucleotides) with metal ions at 37° in 10 mM phosphate (pH 7.4). The reactions were terminated by adding 1 ml of 2% H_3PO_4 and 1 ml of 0.75% TBA solution. The mixture (4 ml) was heated for 20 min in boiling water and cooled.

some thiobarbituric acid (TBA) reactive aldehydes which increased with time. In the presence of hydralazine, however, the formation of TBA reacting aldehydes was slightly stimulated and reached a maximum in about 20 min of incubation. Similar results were also obtained with Fe^{3+}. The addition of Cu^{2+} alone also produced a significant amount of aldehydes which was enhanced in the presence of the drug. EDTA-bound Fe was also effective in producing aldehydes. In fact, EDTA-bound Fe^{2+} stimulated the formation of aldehydes from DNA. Our observations are consistent with recent observations of Brawn and Fridovich [21] who have shown that EDTA-bound Fe is effective in degrading DNA in a reaction mediated by $O_2^{\cdot-}$, $OH^{\cdot}/H_2O_2$. EDTA neither inhibited nor stimulated aldehyde formation in the presence of Cu^{2+}. While the presence of SOD significantly increased aldehyde formation, catalase was inhibitory suggesting that $H_2O_2/OH^{\cdot}$ radicals and not $O_2^{\cdot-}$ were responsible for the aldehyde formation from DNA.

DISCUSSION

Hydralazine binds to native DNA [12]. Using spin-trapping techniques, our studies confirm this binding of hydralazine to double-stranded DNA. The binding most probably involves the intercalation, i.e. the insertion of the ring structure of the hydralazine molecule such that the hydrazine moiety is unavailable for reaction with metals or $O_2^{\cdot-}$. Other binding mechanisms, e.g. ionic binding at the phosphate groups of DNA, are not expected to inhibit this metal ion catalyzed reaction. Furthermore, the observed decrease in DMPO-hydralazyl adduct formation in the presence of added DNA cannot be due to decreased availability of DMPO since a large excess of DMPO is present in the reaction mixture. However, covalent binding of hydralazyl or hydralazyl-derived radicals to DNA is also possible. The single-stranded nucleic acids, RNA and heat-denatured DNA, are not good substrates for hydralazine. These observations confirm earlier results of Eldridge *et al.* [12] who showed that hydralazine binds to native DNA more effectively than denatured DNA. The nature of the binding sites in DNA for hydralazine is not known at this time.

Our studies also show that, in the presence of metal ions and macromolecules, hydralazine stimulated the formation of $O_2^{\cdot-}/OH^{\cdot}$ radicals. Although no $O_2^{\cdot-}$ radicals could be trapped directly in the presence of macromolecules and metal ions at pH 10.0, a significant stimulation of $OH^{\cdot}$ radicals was evident. Furthermore, in incubations carried out at pH 7.4 with Cu^{2+} and macromolecules, $O_2^{\cdot-}$ was readily formed from hydralazine. The formation of the DMPO–OOH adduct in the presence of Cu^{2+} suggests that Cu^{2+} complexes with the oxygen radicals. Cu^{2+} is known to form superoxide–Cu^{2+} complexes in aprotic solvents and, as such, it has been proposed to mimic the actions of superoxide dismutase [25, 26]. Recently, Saryan *et al.* [27] have trapped stable Cu^{2+}–OOH/OH complexes under physiological conditions. It seems, thus, that Cu^{2+}–ligands somehow stabilize this DMPO–OOH complex. Alternatively, Cu^{2+} may enhance the rate of the reaction of $O_2^{\cdot-}$ with DMPO which is known to be slow [28].

Hydralazine stimulated $OH^{\cdot}$ formation in the presence of nucleic acids. Furthermore, hydralazine–metal ions also induced some aldehyde formation which was inhibited by catalase. In contrast, SOD greatly stimulated aldehyde formation. This is due to the formation of H_2O_2 which has been shown to accumulate in the presence of SOD during metal ion catalyzed oxidation of hydrazines [14]. Either H_2O_2 or $OH^{\cdot}$ radicals formed from H_2O_2 in the presence of metal ions may cause aldehyde formation in DNA. H_2O_2 in the presence of metal ions induces aldehyde formation in DNA [29], and the formation of alde-

hydes in DNA by bleomycin–Fe^{2+} complexes has been shown to degrade DNA [22]. Thus, it is possible that hydralazine-induced aldehyde formation in DNA may also lead to DNA degradation.

The molecular mechanism of hydralazine-induced toxicity (induction of SLE and carcinogenicity) is not clear at this time. However, the present studies suggest that binding of hydralazine to nucleic acid through an intercalative mechanism may contribute to its toxicity in a manner similar to other intercalating agents [30]. Moreover, the oxidative metabolism of hydralazine catalyzed by the metals or $O_2^{\cdot-}$ generated by xanthine oxidase through free radical intermediates and the consequent stimulation of $O_2^{\cdot-}$, $OH^{\cdot}/H_2O_2$ formation may play a significant role in hydralazine toxicity. Since xanthine oxidase is present in nuclei [31], formation of the hydralazyl radical at the site of DNA residency is also possible. Either the hydralazyl radical or the reactive secondary radicals produced from reaction with oxygen may then damage DNA or other nuclear structures. Oxygen radicals have been implicated in a number of toxic manifestations including carcinogenesis [19, 20] and DNA strand breaks [21, 32]. Our studies do show the formation of $O_2^{\cdot-}$, $OH^{\cdot}/H_2O_2$ from hydralazine and that $OH^{\cdot}/H_2O_2$ are involved in aldehyde formation in DNA. Repeated insults from hydralazine treatment may cause irreversible damage to nucleic acids which may enhance its carcinogenic potential.

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