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# HYDROXYL FREE RADICAL ADDUCT OF DEOXYGUANOSINE: SENSITIVE DETECTION AND MECHANISMS OF FORMATION

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DNA or 2-deoxyguanosine reacts with hydroxyl free radical to form 8-hydroxy-deoxyguanosine (8-OH-dG). We found that 8-OH-dG can be effectively separated from deoxyguanosine by high pressure liquid chromatography and very sensitively detected using electrochemical detection. The sensitivity by electrochemical detection is about one-thousand fold enhanced over optical detection. Utilizing deoxyguanosine in bicarbonate buffer it was found that ferrous ion, but not ferric ion, was effective in forming 8-OH-dG. The hydroxyl free radical scavenging agents, thiourea and ethanol, were very effective in quenching Fe(11) mediated 8-OH-dG formation, but superoxide dismutase had very little effect.

Key words: oxygen free radical, hydroxyl free radical adduct, DNA damage, 2-deoxyguanosine hydroxylation, HPLC electrochemical detection, hydroxyl radical generation system.

## INTRODUCTION

Oxygen free radicals have been implicated as etiological agents in several disease processes[1] including tumor promotion,[2] aging,[3] ischemic damage to tissues[4] and in the toxic action of some drugs.[5] Oxygen free radicals are known to induce damage to DNA yielding strand breaks,[6] as well as producing specific modified DNA bases such as 5-hydroxymethyluracil[7,8] and thymine glycol.[9] Recently, Kasai and Nishimura[10] demonstrated that various systems known to produce the hydroxyl free radical reacted with either DNA or deoxyguanosine to yield the C-8 hydroxylated nucleoside, 8-hydroxy-deoxyguanosine (8-OH-dG). We have been attempting to develop more sensitive methods of detecting oxygen free radicals *in vivo* and have demonstrated success[11,12] using high pressure liquid chromatography with electrochemical detection (LCED). This technique has the advantages of specificity of separation coupled with specificity and sensitivity of electrochemical detection. The present communication

reports for the first time that 8-OH-dG can be very sensitively detected using the LCED technique. This finding may make it possible to monitor the formation and removal of this modified nucleoside *in vivo*. We also report here some observations relevant to understanding the mechanism of formation of the C-8 hydroxylated nucleoside from deoxyguanosine.

## MATERIALS AND METHODS

The LCED apparatus has been described previously.[11,12] The analytical column utilized in the experiments reported here was a Beckman Ultrasphere ODS 5 $\mu$m, 4.6 mm × 150 mm. The mobile phase was 0.0125 M citric acid, 0.025 M sodium acetate, 30 mM NaOH, 10 mM acetic acid with a final pH of 5.1 to which was added 150 ml methanol per liter. The mobile phase was filtered and vacuum degassed as described before[11,12] and the flow rate was 0.5 ml/min. The column utilized to isolate larger preparative amounts of 8-OH-dG was a Whatman 4P partisil Magnum 9 ODS-3 10 mm × 500 mm operating at a flow rate of 1.5 ml/min. Preparative amounts of 8-OH-dG was synthesized using a Fenton system as described by Kasai and Nishimura.[10] Kasai and Nishimura kindly supplied 8-OH-dG as a standard for comparison purposes. Mass spectroscopy was accomplished on the trimethylsilyl derivative of 8-OH-dG utilizing direct probe injection into a Hewlett-Packard 5985 instrument. The mass spectrum of trimethylsilated 8-OH-dG yielded distinctive peaks at M/Z values of 643 ($M^+$), 628 ($M^+$-$CH_3$), 383 (base + H) and 368 (base + H − $CH_3$), as was reported by Kasai and Nishimura.[10]

We examined the role of $\dot{O}H$ in the generation of 8-OH-dG from deoxyguanosine utilizing the UV-$H_2O_2$ and the ADP/Fe(II)/$H_2O_2$ systems described previously.[12] A NaCl/$NaHCO_3$ buffer (100 mM/25 mM, pH 7.4) was used to solubilize deoxyguanosine and examine the role of $\dot{O}H$ and Fe(II) in the formation of 8-OH-dG. ADP as the Na salt and deoxyguanosine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade or better.

## RESULTS

Figure 1 demonstrates three important facts. They are:

a) the LCED system clearly separates deoxyguanosine from 8-OH-dG;

b) deoxyguanosine is not detected electrochemically, but in contrast 8-OH-dG is clearly detected electrochemically (oxidation mode); and,

c) the electrochemical detection system is much more sensitive than the optical absorbance method.

The traces shown in Figure 1 were obtained with the optical detector placed in series with the electrochemical detector. The optical detector was operating at nearly maximum sensitivity; whereas, the electrochemical detector system was operating at the most insensitive setting available; despite this fact, the trace obtained could not be registered on the recorder.

Figure 2 presents the hydrodynamic voltammogram of 8-OH-dG in reference to the Ag/AgCl electrode. The oxidation potential at half-maximum height is 0.375 volts.

We have found, as did Kasai and Nishimura,[10] that 8-OH-dG is formed from

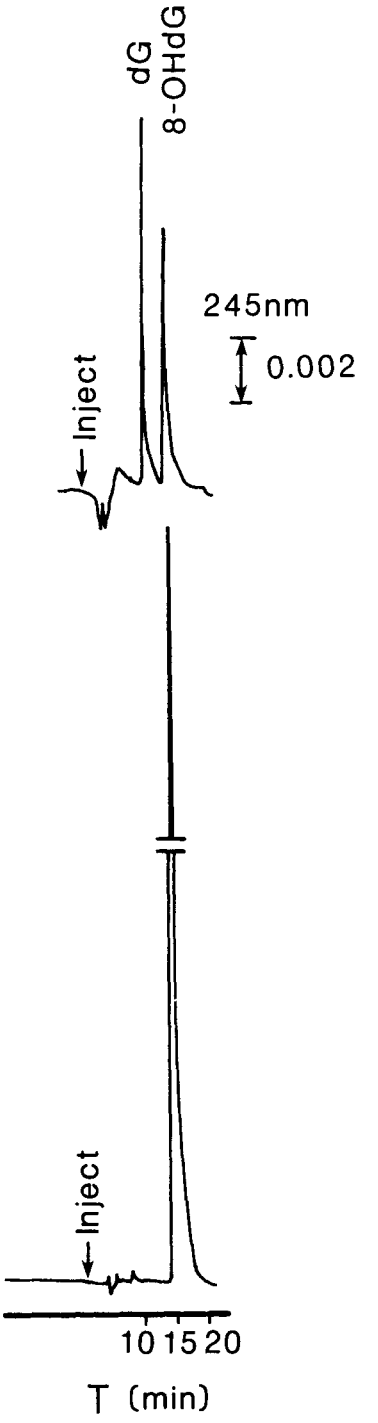


FIGURE 1 Comparison of optical detection (upper trace) with electrochemical detection (lower trace) of deoxyguanosine (dG) and 8-hydroxy-deoxyguanosine (8-OH-dG). The time of injection is noted, as well as elution time. The optical detector was set at nearly maximum sensitivity (AA = 0.002, as noted) and the electrochemical detector set at the most insensitive response were arranged in series.

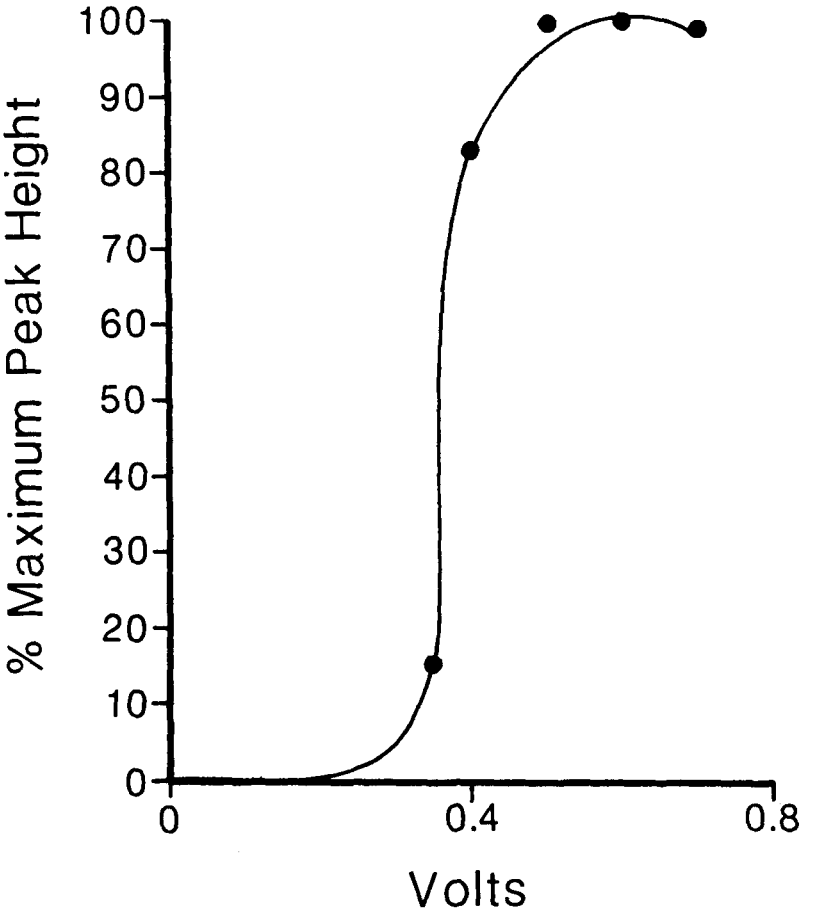


FIGURE 2 The hydrodynamic voltammogram of 8-hydroxy-deoxyguanosine as compared to the Ag/AgCl reference electrode.

deoxyguanosine in several systems known to produce OH, such as the ADP/Fe(II)/$H_2O_2$ systems. Interestingly, we found that the addition of ferrous chloride to the bicarbonate buffer system containing deoxyguanosine produced 8-OH-dG almost as effectively as in the ADP/Fe(II)/$H_2O_2$ system and, hence, we have examined the Fe(II)/deoxyguanosine/bicarbonate buffer system in more detail. Figure 3 shows the amount of 8-OH-dG formed in this system as a function of added deoxyguanosine. The half-maximum amount of 8-OH-dG formation occurred at about 65 $\mu$M deoxyguanosine. The ferrous ion was added as ferrous chloride (nitrogen sparged in 0.0012 N HCl) at a final concentration of 500 $\mu$M. Within 30 sec. after adding Fe(II), the reaction was complete and a portion of the sample (20 $\mu$l) was injected into the LCED system for analysis of the 8-OH-dG present. No 8-OH-dG was formed if dilute HCl without $FeCl_2$ was added. Also, very little, if any, 8-OH-dG was formed if Fe(III) was substituted for Fe(II). Thus, Fe(II) is essential for formation of 8-OH-dG from deoxyguanosine.

We have tested the effects of thiourea and ethanol, two effective ȮH scavengers, on the formation of 8-OH-dG from deoxyguanosine in the Fe(II)-bicarbonate buffer system. The results are shown in Figures 4 and 5, respectively. The results show that thiourea and ethanol effectively inhibit 8-OH-dG formation with half-maximum concentrations of about 60 $\mu$M and less than 1 mM, respectively. The effect of Cu-Zn superoxide dismutase (SOD) is shown in Figure 6. SOD appeared to have very little, if any, effect on formation of 8-OH-dG from deoxyguanosine in the Fe(II)-bicarbonate buffer system.

## DISCUSSION

Oxygen free radical attack on DNA may lead to strand breakage and/or formation of specific modified bases such as thymine glycol, hydroxymethyluracil or, as shown by Kasai and Nishimura,[10] formation of 8-hydroxyguanosine. Oxygen free radicals damage to DNA may be repaired depending upon the type and extent of damage, but nothing is known yet regarding the repair of 8-hydroxyguanosine or, in fact, if this damage is produced *in vivo*. The present report shows that 8-OH-dG can be detected at very low levels (subpicomoles) using the LCED technique. Based upon the absorptivity of 8-OH-dG at 245 nm (E = 12,300), as given by Kasai and Nishimura,[10] and the demonstrated sensitivity of the LCED technique,[11,12] it appears that the latter has about one-thousand fold greater sensitivity than optical detection. This enhanced sensitivity should make it more readily possible to examine the formation and repair of 8-OH-dG in biological model systems.

Regarding the mechanism of formation of 8-OH-dG, it was shown by Kasai and Nishimura[10] that many reducing agents including hydrazine, hydroxylamine and ascorbic acid mediated formation of 8-OH-dG, but not as effectively as the Udenfriend ȮH generating system. We have also found that ȮH generating systems will form 8-OH-dG from deoxyguanosine. Interestingly, we also found that Fe(II) alone readily mediates 8-OH-dG formation from deoxyguanosine. This reaction was inhibited by thiourea and ethanol, both widely used as ȮH scavenging agents. The exact mechanism of Fe(II) mediated formation of 8-OH-dG needs further investigation. It is possible that Fe(II) and deoxyguanosine form a 1:1 complex and that this is required as a first step in a concerted process leading to the eventual formation of 8-OH-dG. We are investigating these processes in greater detail at the present time.

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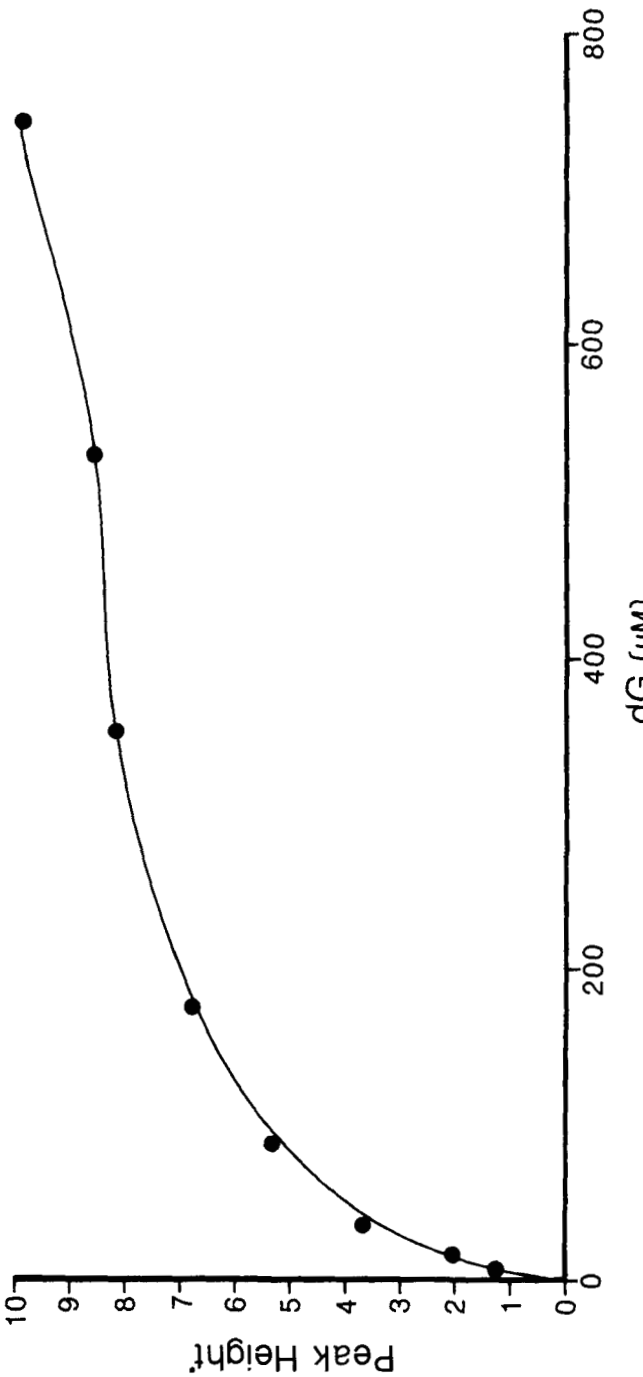


FIGURE 3 The amount of 8-OH-dG formed (peak height) versus deoxyguanosine present in a bicarbonate buffer system to which ferrous chloride (500 $\mu$M) was added.

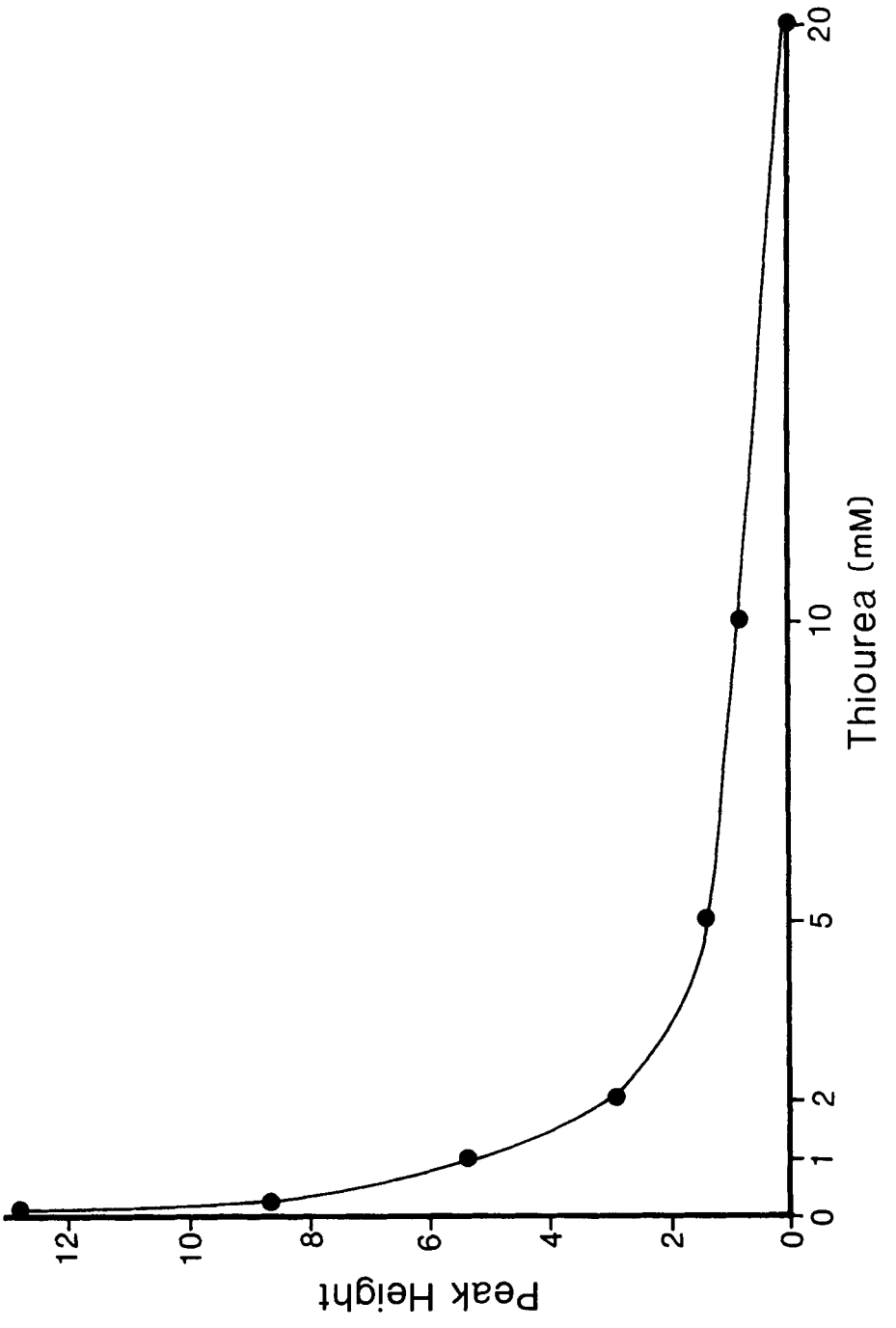


FIGURE 4 Thiourea inhibition of 8-OH-dG formation from deoxyguanosine (750 $\mu$M) as mediated by ferrous chloride in a bicarbonate buffer system.

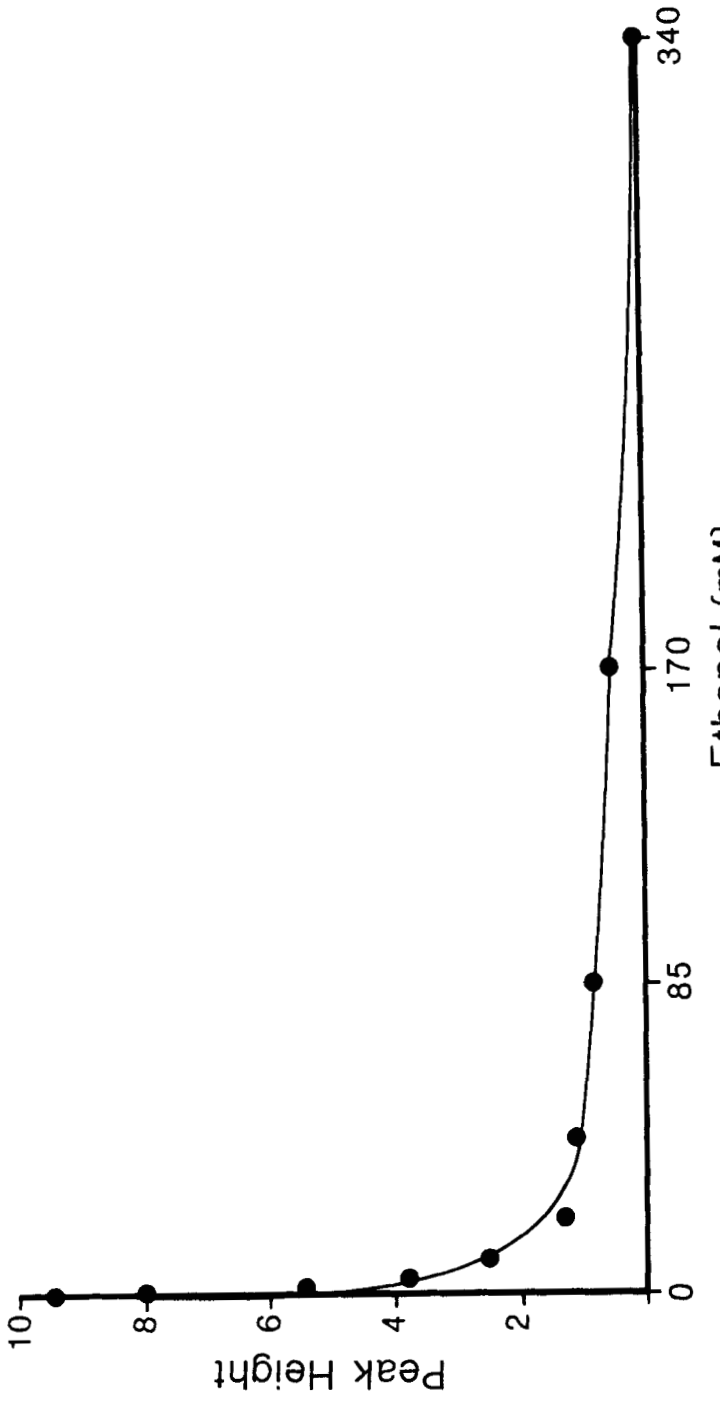


FIGURE 5 Ethanol inhibition of 8-OH-dG formation from deoxyguanosine (750 $\mu$M) as mediated by ferrous chloride in a bicarbonate buffer system.

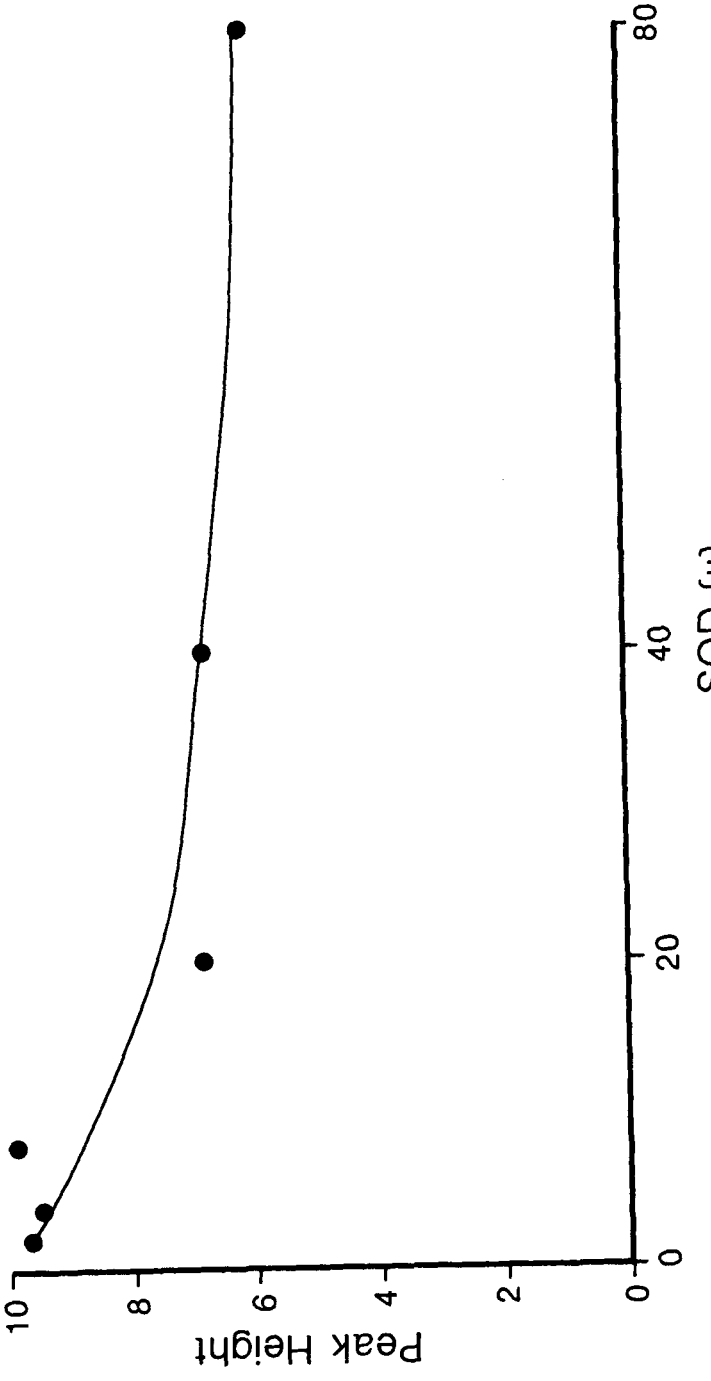


FIGURE 6 The effect of Cu-Zn superoxide dismutase (SOD) on formation of 8-OH-dG from deoxy- guanosine (750 $\mu$M) as mediated by ferrous chloride in a bicarbonate buffer system.

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