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**IRON-DITHIOTHREITOL DEPENDENT PRODUCTION OF
8-HYDROXY-2'-DEOXYGUANOSINE IN DNA**

by

David Allan Cook

Thesis submitted in partial fulfillment
of the requirements for the degree

of

UNIVERSITY HONORS
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in

Chemistry

UTAH STATE UNIVERSITY
Logan, UT

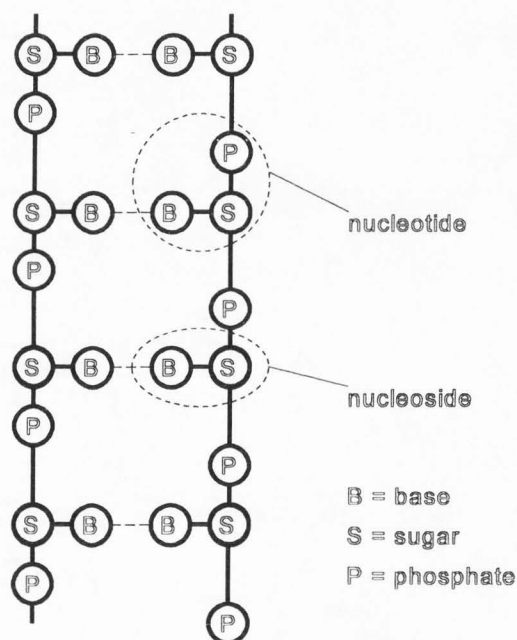
1995

Iron-Dithiothreitol Dependent Production of 8-hydroxy-2'-deoxyguanosine in DNA

Oxidative damage to DNA in a metal-thiol system was studied. Calf thymus DNA was incubated in an iron(III)-dithiothreitol system prepared in various buffers. Damage was measured by monitoring the production of 8-hydroxy-2'-deoxyguanosine. DNA oxidation was proportional to the concentration of dithiothreitol (DTT) in HEPES and tris(hydroxymethyl)aminomethane (Tris) buffers while it was only roughly related to DTT concentration in sodium chloride. Incubation in potassium phosphate buffer produced no damage. The results indicate that hydroxyl radicals may be generated by the iron-DTT system in HEPES, Tris, and sodium chloride, while in the phosphate buffer an iron-phosphate complex may be formed which limits the participation of the iron in the redox cycle.

Deoxyribonucleic acid, commonly known as DNA, contains an organism's genetic code -- the blueprint of life. A DNA molecule is very similar to a ladder in shape. If the "ladder" were cut up the middle to separate the sides and then divided into segments each containing one rung, each segment would be composed of three parts. The side of the ladder would be made of a sugar (*deoxyribose*) attached to a phosphate group, with the phosphate of one segment binding to the sugar of the next to form the DNA "backbone."

Each half "rung" would be one of four *bases*: adenine, thymine, guanine, and cytosine. A base and a sugar together form a unit called a *nucleoside*, and a nucleoside with a phosphate is called a *nucleotide*. Each nucleoside is named for the base to which it is attached. For example, guanosine and deoxyribose together are called *deoxyguanosine*.

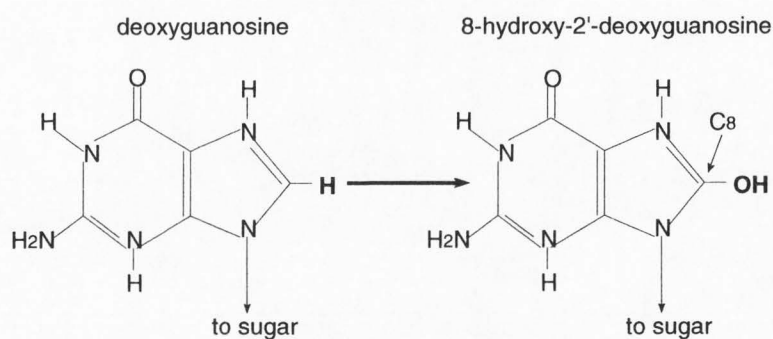


The "language" of the code is defined by the sequence of the bases along the DNA backbone like rungs on a ladder. The language is interpreted when the DNA code is translated into a protein. A gene is the sequence of bases that codes for the production of a specific protein. If this sequence is altered the corresponding protein changes accordingly, often at a cost in its effectiveness. For example, a single change in one base in the gene for hemoglobin can result in a blood disease such as sickle cell anemia or thalassemias. When a cell divides the DNA is replicated so that each new cell can have a copy. Thus, any changes made to DNA will be passed on to the following generations.

One way to alter the base sequence of DNA is to damage or modify a base. Although a damaged base is often repaired by enzymes, on occasion the restored base may not be the same as the original. If left unrepaired the base may be confused with another during replication. In either case, when the cell divides the DNA strand is copied incorrectly -- and a permanent change is made in the DNA code. Damage to and modification of DNA bases has been shown to have a high correlation with mutation and cancer (1).

DNA can be damaged by both radiation and chemical reactions. The chemical reactions involved are called oxidative or redox reactions because one molecule is chemically *reduced* while another (in this case the DNA) is *oxidized*. One of the major products of oxidative DNA damage is 8-hydroxy-2'-deoxyguanosine (8-OH-dG)^a (2), a modification of the standard DNA nucleoside deoxyguanosine. In the reaction, the hydrogen atom is replaced by a hydroxyl group (HO) as seen in the reaction below:

a. Abbreviations. 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; ·OH, hydroxyl radical; RSH, any thiol; Tris, tris(hydroxymethyl)aminomethane; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT dithiothreitol; HPLC, high performance liquid chromatography; deoxyguanosine (dG).



Electrochemical detection allows easy detection of 8-OH-dG by high performance liquid chromatography (HPLC)^a (3), making 8-OH-dG a common measure of oxidative damage.

The hydroxyl radical ($\cdot\text{OH}$)^a has been implicated as one of the major sources of damage to DNA and other cellular components (1). Menghini and Martins (4) propose that $\cdot\text{OH}$ may be the ultimate DNA damaging agent and that other DNA damaging agents are converted to $\cdot\text{OH}$ before attacking the DNA strand. For this reason, the sources of hydroxyl radical have become a topic of research in recent years. Iron has long been known as a mediator in the production of hydroxyl radical, and has more recently been linked with DNA damage and cancer (5). One way in which iron can catalyze the production of $\cdot\text{OH}$ is in a metal-catalyzed reaction with thiols.^b

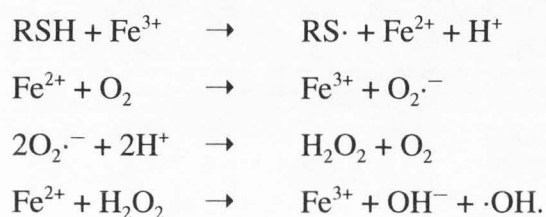
Thiol-containing compounds are generally considered to have a positive effect on biological systems because they scavenge free radicals, protecting biomolecules from damage. However, the same reducing ability that confers this antioxidant character can itself cause damage. Thiols can react with a transition metal ion such as copper or iron to produce thiyl radicals. The thiyl radical can continue to react, producing damaging oxygen radicals^c (6, 9-10).

b. A thiol is a group of sulfur-containing compound similar to alcohols, but whereas an alcohol contains an $-\text{OH}$ group, a thiol contains an $-\text{SH}$ group.

c. Thiols cannot react directly with dioxygen (O_2) because, while thiols are in a singlet state, dioxygen is in a triplet state. The reaction is "forbidden."

Iron has been shown to be particularly potent in effecting the production of both thiyl and oxygen radicals in a thiol system (7). When a thiol or dithiol (RSH)^a such as dithiothreitol (DTT)^a is mixed with ferric iron (iron(III) or Fe³⁺) both thiyl radical (RS·) and reduced iron (iron(II) or Fe²⁺) can form (8). The reaction of molecular oxygen with reduced iron forms superoxide radical (O₂^{·-}) which can then react to produce the hydroxyl radical, a potent oxidant.

These reactions constitute the *redox cycle*^d shown below (9):



Park and Floyd (10) have recently shown that this iron/thiol/oxygen system produces both strand breaks and 8-OH-dG in DNA. They also give evidence that the hydroxyl radical is the root cause of this damage.

When a metal ion is in solution, it is surrounded or *chelated* by other molecules known as *ligands* to form a *complex*. The surrounding molecules may simply be water or they may be other components of the solution. The nature of the ligand can greatly affect the reactivity of the metal ion. The production of radicals in metal-thiol systems varies greatly with the chelation or lack thereof of the metal ion, and also with the nature of the ligand (7, 11). It would seem that certain ligands shield the iron from reaction with the thiol.

d. A *redox* or *reduction-oxidation cycle* is a series of chemical reactions in which a compound goes through a change in its electronic state (is oxidized or reduced) and then is regenerated. For example, in the reaction series shown above, iron is reduced (its electric charge goes from 3+ to 2+) while the DTT is oxidized. Both, however, are recovered in the end of the cycle.

In biochemical research, an attempt is made to simulate the normal physiological state of the cell. *Buffers* are added to test solutions to keep them at a desired pH, among other things. No buffer can duplicate the complex environment found inside the cell, however, and the results observed *in vitro* may often vary considerably from the actual events *in vivo*. Although research has been done on the iron-thiol system described above, the influence of different buffers on the system has not been explored. It was thought that perhaps different buffers could act as ligands that complex with and alter the ability of the iron to redox cycle.

In this study, the effect of various buffers on the metal-catalyzed thiol oxidation reaction was investigated using ferric iron and DTT. Three representative buffers commonly used in *in vitro* research and a dilute salt solution of sodium chloride were selected. The amount of 8-OH-dG produced was measured as an indication of DNA damage. It was proposed that the buffers would vary in their ability to promote iron redox cycling, while in the chloride solution the iron would be stabilized in the ferrous form and further cycling prevented. This would be observed as varying amounts of DNA damage. The results show that under the conditions used, iron(III) and DTT reacted in HEPES^a, Tris^a, and sodium chloride to promote significant DNA damage, while little damage was observed in the potassium phosphate buffer.

Materials and Methods

Materials. Potassium phosphate, tris(hydroxymethyl)aminomethane (Tris), sodium chloride, and ferric chloride were obtained from Mallinkroft. Calf thymus DNA, dithiothreitol (DTT), HEPES, phosphodiesterase II, and deoxyribonuclease I (DNase I) were obtained from Sigma. Phosphodiesterase (from *crotallus durissis*) and alkaline phosphatase were obtained from Boehringer Mannheim. Stocks of HEPES, Tris, potassium phosphate, and sodium chloride (all 50 mM, pH 7.0) were prepared and ultrafiltered. Stock solutions of calf thymus DNA were prepared in each of these buffers. Dithiothreitol and ferric chloride were prepared immediately before use in the buffer of choice. The HPLC mobile phase consisted of 17% methanol and 83% 50 mM phosphate at pH 5.5, and was Chelex treated before use.

Methods. The reaction was carried out in the buffer of choice (final pH 7.0), which contained 0.25 mg/ml calf thymus DNA, 30 μ M ferric chloride, and varying amounts of DTT. The mixture was incubated under aerobic conditions for 2 hr. at 37°C in a shaking water bath. After incubation, the DNA was precipitated with sodium acetate and ethanol and resuspended in 40 mM Tris/10 mM MgCl₂ pH 8.5. It was then digested with DNase I, alkaline phosphatase, phosphodiesterase II (calf spleen) and phosphodiesterase (from *crotallus durissis*) for 1 hr. The nucleases were removed by filtration. The resulting mixture was injected into the HPLC. Elution of 8-OH-dG was detected using an electrochemical detector. A uv detector (set at 254 nm) was used to detect deoxyguanosine (dG)^a. A Beckman System Gold HPLC was used; the column was an Alltech (250 x 4.6 mm). The molar ratio was determined using a standard curve. All results presented are the average of experiments performed in triplicate.

Results

Figure 1 shows the amount of 8-OH-dG formed when ferric iron and DNA were incubated with varying amounts of DTT in the solutions indicated. Results are presented as the ratio of 8-OH-dG to deoxyguanosine (dG)^a. Floyd (1) has stated that the normal physiological range of 8-OH-dG is 0.5-2.0 8-OH-

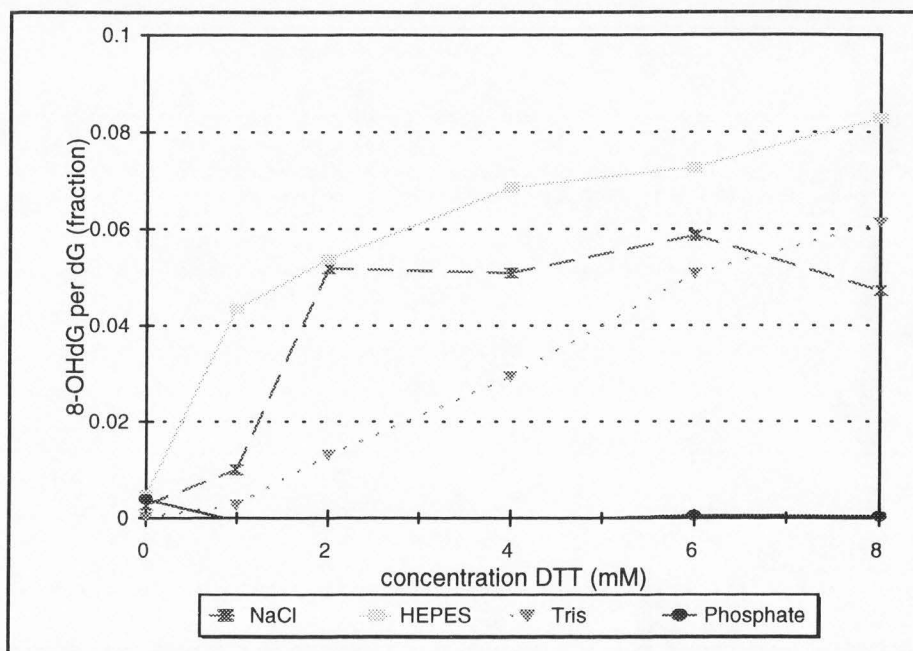


Figure 2. Concentration-dependent formation of 8-OH-dG in DNA by an iron(III)/DTT/oxygen system. All buffers were 50 mM, with final pH 7.0. The reaction mixture contained 0.25 mg/ml calf thymus DNA, 30 μ M FeCl₃, and the DTT indicated. Incubation was performed as described in the Methods section. Normal physiological fraction of 8-OH-dG is 0.5-2.0 8-OH-dG/10⁵ dG.

dG/10⁵ dG, although some base modification may occur in the purification and handling of DNA.

Significant base damage -- up to 10,000 times the normal physiological range -- was detected

when ferric iron and DTT were incubated in HEPES, Tris, and sodium chloride. The reaction in phosphate buffer showed no damage.

Discussion

When DNA was incubated with ferric chloride and DTT the amount of base damage observed varied widely from buffer to buffer. In the potassium phosphate buffer, virtually no base modification was seen. It is possible that under the experimental conditions, a phosphate-iron complex was formed which inhibited the redox cycling of the iron between the iron(III) and iron(II) forms. Without an electron acceptor the DTT could not redox cycle and no damaging radicals were formed.

The iron(III) complex formed in the HEPES and Tris buffers, on the other hand, permitted the redox cycling of the iron and the subsequent modification of DNA bases. It is interesting to note the 25% difference in activity between reactions in the two buffers. This may be due to a difference in the iron-buffer complex formed which modifies its ability to redox cycle. It may also be due to differences in the ability of the buffer to scavenge and remove hydroxyl radicals before they react with and damage DNA strands.

That sodium chloride should promote oxidation in the system was a result diametrically opposed to that predicted. It was supposed that in the absence of iron-buffer complexes the iron(III) would be reduced and stabilized in the iron(II) form by the DTT. This redox cycling-inert species would not react with the oxygen, and no DNA damage would be observed. Significant DNA damage was seen, however, which was roughly related to the concentration of DTT. Another mechanism of DNA damage may be the cause. Sakurai *et al.* (12) incubated DNA with ferric chloride and glutathione (another thiol) and obtained results suggesting that free

iron complexes with DNA where it redox cycles with the thiol and produces base-damaging hydroxyl radicals. In the present study a similar DNA-iron complex may form at the DNA concentrations used and in the absence of buffer. Personal communication with Nate Spear confirmed that at lower DNA concentrations a much lower fraction of deoxyguanosine is converted to 8-OH-dG. Iron-DNA redox cycling may be the source of 8-OH-dG in the sodium chloride mixture.

It has been shown (13) that thiols, particularly glutathione, do occur within the cell at concentrations and locations such that DNA damage via the metal catalyzed oxidation scheme described here could occur. However, the effect of the reaction may be mediated by several factors. Cellular enzymes, including oxygen and thiol radical scavengers, can remove potentially damaging species. Yim *et al.* (11) showed that a thiol-specific antioxidant enzyme removed thiol radicals from solution before they could damage biomolecules directly or produce more reactive radicals. The wide variation in DNA base damage between buffers as demonstrated in this study may also have implications when considering an actual biological system. Complexation effects *in vivo* may reduce or eliminate the ability of a metal ion to redox cycle with a thiol. Further study of the iron/thiol/oxygen system is necessary to determine its role in *in vivo* DNA damage and its impact on mutation and cancer.

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