GENERATION OF DNA-DAMAGING REACTIVE OXYGEN SPECIES VIA THE AUTOXIDATION OF HYDROGEN SULFIDE UNDER PHYSIOLOGICALLY RELEVANT CONDITIONS

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

GENERATION OF DNA-DAMAGING REACTIVE OXIDATION SPECIES VIA THE AUTOXIDATION OF HYDROGEN SULFIDE UNDER PHYSIOLOGICALLY RELEVANT CONDITIONS

Presented by Marjorie A. Hoffman,
a candidate for the degree of Master of Science,
and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Kent Gates

Dr. Tim Glass

Dr. Carol Deakyne

To my Family

I would like to dedicate this work to my family near and far, for all their love and support. To my parents, Mark and Maureen Hoffman, who always encouraged me in whatever I wanted to pursue. To my brother, Rob, for always being there when I needed him. To my cousin, Paul, for reminding me that it is okay when the road takes a turn you did not expect. To all my aunts, uncles, cousins, grandparents, and friends who have supported and encouraged me throughout my life.

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TABLE OF CONTENTS

Acknowledgements……………………………………………………………………..ii

Table of Contents ………………...……………………..…………………………......iii

List of Figures ………………………………...……………………………………..v

List of Schemes ……………………………………...………………………...……vi

List of Tables …………………………………..……………………………….vii

Abstract ……………………………………………………………………………...…viii

Chapter 1: Introduction to the Biological Activity of Hydrogen Sulfide

1.1 Introduction ……………………………………………………..............................1

1.2 Generation of H_2S …………………………………...…………………………..2

1.2.1 Endogenous Sources of H_2S …………………………………………….....2

1.2.2 Endogenous Concentration of H_2S ……………………………………..…4

1.2.3 Inhibition of H_2S Production …………………………………………...…5

1.2.4 Exogenous Sources of H_2S ……………………………………………….…6

1.3 Removal of H_2S from the Body …..…………………………………………….………8

1.4 Biological Effects of H_2S ………………….……………………………………….…8

1.5 References ………………………………………………………………………...…...10

Chapter 2: Generation of Reactive Oxygen Species via the Autoxidation of H_2S under Physiologically Relevant Conditions

2.1 Introduction ……………………………………………………………………….....13

2.2 DNA Strand Cleavage by H_2S ………………………………………………………15

2.3 Mechanism of DNA Strand Cleavage by H_2S ……………..……………………..17

2.4 Investigating DNA Strand Cleavage by H_2S Decomposition Products ……...……...20

2.5 Effect of Thiols on DNA Strand Cleavage by H_2S …………………………..……21

2.6 Discussion ……………………………………………………………………...23

2.7 Materials and Methods ……………………………………………………….....26

2.7.1 Chemicals and Reagents ………………………………………………...26

2.7.2 Cleavage of Plasmid DNA by H_2S ……………………………………...26

2.8 References ……………………………………………………………………...28

LIST OF FIGURES

CHAPTER 1

Figure 1. Inhibitors of CSE and CBS ……………………………………………………….5

Figure 2. Select H_2S Generating Compounds ……………………………………..……7

Figure 3. Select Phytochemical Sources of H_2S …………………………………...…..8

CHAPTER 2

Figure 1. Various Sulfur Containing Molecules ………………………………………...14

Figure 2. DNA Strand Cleavage by DNA ……………………………………………....17

LIST OF SCHEMES

CHAPTER 1

Scheme 1. Dissociation of H_2S ………………………………………………………...…2

Scheme 2. Synthesis of H_2S by CSE and CBS ………………………………………...3

Scheme 3. Synthesis of H_2S by CAT and 3-MPST ………………………………………4

CHAPTER 2

Scheme 1. Reactions involved in the autoxidation of H_2S …………………...………19

Scheme 2. Generation of protein persulfides from thiols via the oxidation of H_2S ……..25

LIST OF TABLES

CHAPTER 2

Table 1. Effect of additives on DNA cleavage by H_2S ………..……………........18

Table 2. Cleavage of plasmid DNA by H_2S in the presence of various concentrations of iron ………………………...………………………20

Table 3. Cleavage of plasmid DNA by H_2S oxidation products ………………….21

Table 4. Cleavage of plasmid DNA by H_2S in the presence of various thiols ……23

ABSTRACT

Hydrogen sulfide (H_2S) is more commonly known for its toxic properties; however, recently, there has been evidence that this small, gaseous molecule could serve as an endogenous cell-signaling agent. Surprisingly, a number of studies have also provided evidence that H_2S is a DNA-damaging mutagen. Using a plasmid-based DNA strand cleavage assay, we examined the chemical mechanisms of DNA damage by H_2S. We found single-strand DNA cleavage was caused by micromolar concentrations of H_2S. The mechanistic process was studied and was shown to involve the autoxidation of H_2S to generate superoxide, hydrogen peroxide, and ultimately hydroxyl radical, a well-known DNA-damaging agent, via a trace metal-mediated Fenton-type reaction. In the presence of physiological thiol concentrations, DNA strand cleavage by H_2S still occurred. The oxidation byproducts of H_2S, such as thiosulfate, sulfite, and sulfate, do not contribute to DNA strand cleavage. However, the initially generated oxidation products, like persulfide (S_2^{2-}), most likely go through rapid autoxidation reactions, which contribute to superoxide generation. This autoxidation process is of potential relevance to both the genotoxic and cell signaling properties of H_2S.

Chapter 1

Introduction to the Biological Activity of Hydrogen Sulfide

1.1 Introduction

Hydrogen sulfide, H_2S, a colorless gas known for its rotten egg smell, is naturally found in volcanic vents, hot springs, and swamps. Although known since ancient times due to its presence in nature, Swedish chemist Carl Wilhelm Scheele is given credit for its isolation and discovery in 1777.[1] H_2S is denser than air and therefore collects in low-lying areas, which could become dangerous in unventilated areas.[2] Although the gas is detectable to the human nose at concentrations as low as 0.02 – 0.13 ppm, the ability to smell H_2S deadens after a few minutes, making it dangerous to rely on the sense of smell to tell if one is in the presence of dangerous concentrations of H_2S.[3] Exposure to 300 ppm of H_2S can lead to loss of consciousness, with death possibly occurring after 30 min; exposure to 700 ppm can lead to loss of life within minutes.[2]

H_2S is slightly soluble in water, with a solubility of 0.116 M at 20°C, which decreases to 0.0815 M at 37°C.[2] In general when discussing sulfide concentration, 'sulfide' refers to the mixture of H_2S, SH^-, and S^{2-} in solution. At 37°C, the first pK_a of

H_2S is 6.76 and the second pK_a is ~19; therefore, at physiological pH, only about 20% of the sulfide exists as H_2S, the rest is found predominately in the form of SH^- (Scheme 1).[2]

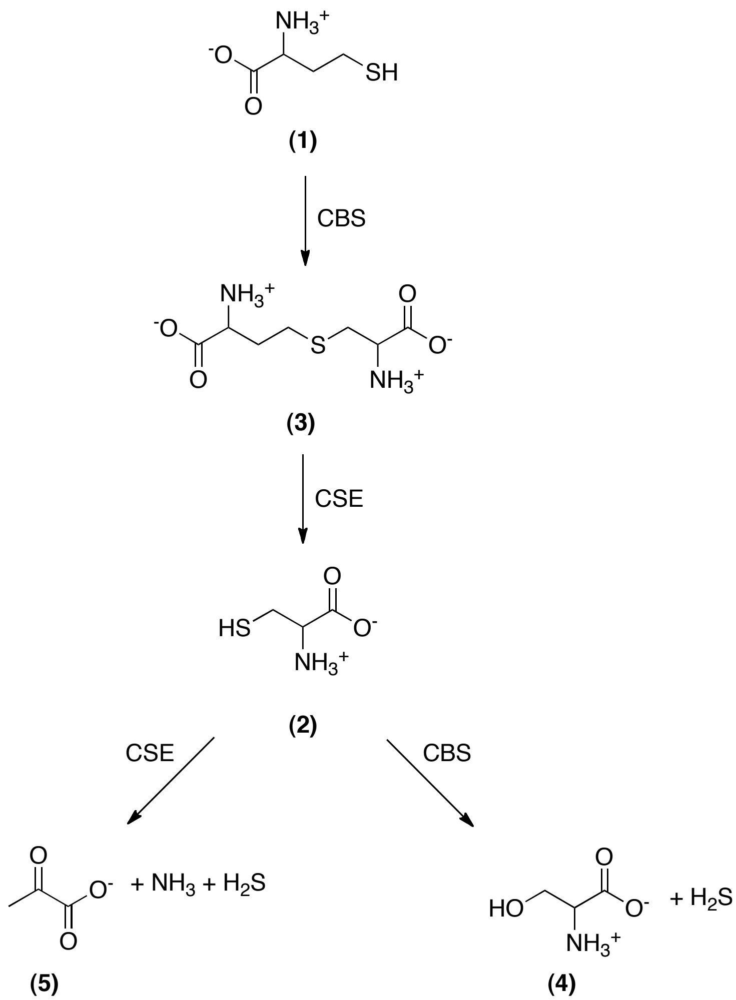
$$H_2S \underset{}{\overset{pK_a = 6.76}{\rightleftharpoons}} SH^- + H^+ \overset{pK_a = 19}{\rightleftharpoons} S_2^- + 2H^+$$

Scheme 1. Dissociation of H_2S, shown with pK_as at physiological pH 7.4.

1.2 Generation of H_2S

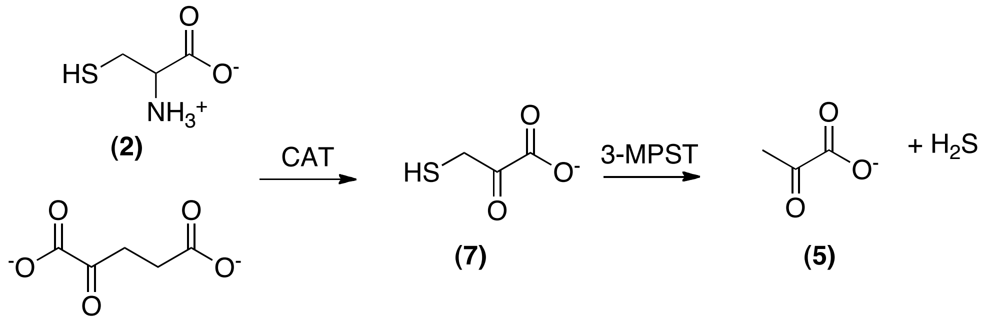
1.2.1 Endogenous Sources of H_2S

Hydrogen sulfide has long been known to be a toxic, noxious gas; however, recently, it has been shown to be an endogenous cell-signaling agent.[4-6] H_2S is produced endogenously by several enzymatic pathways. One major pathway involves cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) from homocysteine (**1**) and cysteine (**2**).[2,4,7-11] Cystathionine (**3**) is produced by CBS from homocysteine via the addition of L-serine (**4**) (Scheme 2).[2, 4, 7-11] **2** is produced by **3** from CSE; CBS then catalyzes the transformation of **2** to **4** and H_2S or CSE can catalyze the transformation of **2** to pyruvate (**5**), ammonia, and H_2S.[2, 4, 7-11]



Scheme 2. Synthesis of H_2S by cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS).

Recently, it has been shown that a second pathway involving 3-mercaptopyruvate sulfur transferase (3-MPST) can also produce H_2S (Scheme 3).[2,7-8,12] Cysteine aminotransferase (CAT) catalyzes the transformation of α-ketoglutarate (**6**) and **2** to 3-mercaptopyruvate (**7**), which is then changed to **5** and H_2S via 3-MPST.[2,7-8,12]



Scheme 3. Synthesis of H_2S by cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfur transferase (3-MPST).

The enzymes involved in H_2S production depend on which part of the cell or body is being investigated. CSE is found in the cytosol[8] and expressed in the cardiovascular system[13], including smooth muscle cells and peripheral tissues,[2,10,14] whereas CBS is usually expressed in the brain and central nervous system.[2,10,13-14] In some places, such as the liver, both CSE and CBS are found.[7,14] 3-MPST is found in both the mitochondria and cytosol and is expressed in the brain, liver, kidney, and aorta.[7,12,14]

Sulfide production in mammalian cells has been measured at 1 – 10 pmol sec^{-1} mg^{-1} of protein, with the highest concentration being found in the brain and liver.[15] Possible non-enzymatic sources of H_2S include the reduction of glutathione (GSH), elemental sulfur, and inorganic or organic poly-sulfides.[8]

1.2.2 Endogenous Concentration of H_2S

Varying concentrations of endogenous hydrogen sulfide ranging from 15 nM to 150 μM have been reported in the literature.[5,10,13] The reported micromolar concentrations of H_2S may not be accurate though, and are probably due to an artifact of the method used to measure sufide.[10,16] A 100 μM solution of H_2S in phosphate buffer gives off the characteristic rotten egg smell of H_2S; if the body contained the proposed micromolar concentrations, an open wound would also produce the same rotten egg smell, which it does not.[2,10] It appears the method used to measure sulfide defines the amount of sulfide detected in samples; higher concentrations of detected sulfide were likely caused by liberated bound sulfide.[10] The endogenous H_2S concentration is now generally accepted to be around 10 – 30 nM.[14,16-18]

1.2.3 Inhibition of H_2S Production

Several inhibitors of H_2S producing enzymes have been used in recent years to analyze the effects of H_2S in vivo.[5,9-10,13] All the inhibitors target pyridoxal phosphate binding sites and have low selectivity. DL-propargylglycine (PAG) and β-cyanoalanine (BCA) both inhibit CSE (Figure 1).[10,13] They have limited cell membrane permeability and perform 50% inhibition at 100 μM and 90% inhibition at 1 mM in rodent models.[10] Inhibitors of CBS include aminooxyacetic acid and hydroxylamine (Figure 1).[5,9-10,13]

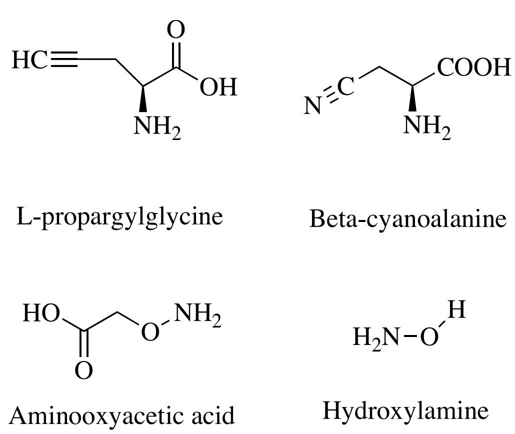


Figure 1. Inhibitors of cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS).

1.2.4 Exogenous Sources of H_2S

There are a number of chemicals that produce hydrogen sulfide under physiological conditions. The most basic methods of producing a sulfide solution involve dissolving either H_2S gas or one of its salts in an aqueous medium; the two most common salts being Na_2S•$9H_2O$ and NaSH•xH_2O.[2] Practically speaking the salts are easier to work with, as they are solids; however, they can contain impurities that must be removed if possible. In this regard Na_2S•$9H_2O$ is the easiest to clean (the outside of the crystals must be washed to remove any sulfur oxidation impurities) and the easiest to use to calculate an accurate concentration of sulfide because the number of hydrides is known as opposed to NaSH•xH_2O.[2,19] A solution of H_2S can be made from the gas by bubbling the gas through deoxygenated solvent.[2] No matter which method is used, impurities can result from the oxidation of SH^-, which are catalyzed by trace metals. The addition of a chelator such as DETAPAC can help prevent autoxidation.[2,19]

Other sources of H_2S include thioacetamide and Lawesson's reagent (Figure 2).[2] Thioacetamide is carcinogenic, highly toxic, and decomposes in water to generate H_2S.[2] Lawesson's reagent, 2,4-bis (4-methoxyphenyl) 1,2,3,4-dithiaphosphetane-2,4-disulfide, undergoes a ring opening, which produce two dithiophosphine molecules that decompose to H_2S.[2] Lawesson's reagent is also toxic and has a foul smell.

The previous compounds release H_2S as soon as the compounds dissolved in solution; to allow for a constant concentration of H_2S instead of a large release of H_2S at the beginning of a reaction, several groups have developed compounds that slowly release over time. One of these compounds is called GYY4137 (Figure 2).[20] Another compound used to produce H_2S is anethole dithiolethione (ADT).[21] The dithiolethione moiety has been added to many nonsterodial anti-inflammatory drugs (NSAIDs) including aspirin, diclofenac, ibuprofen, and naproxen to reduce gastric side effects caused by these NSAIDs (Figure 2).[2,21]

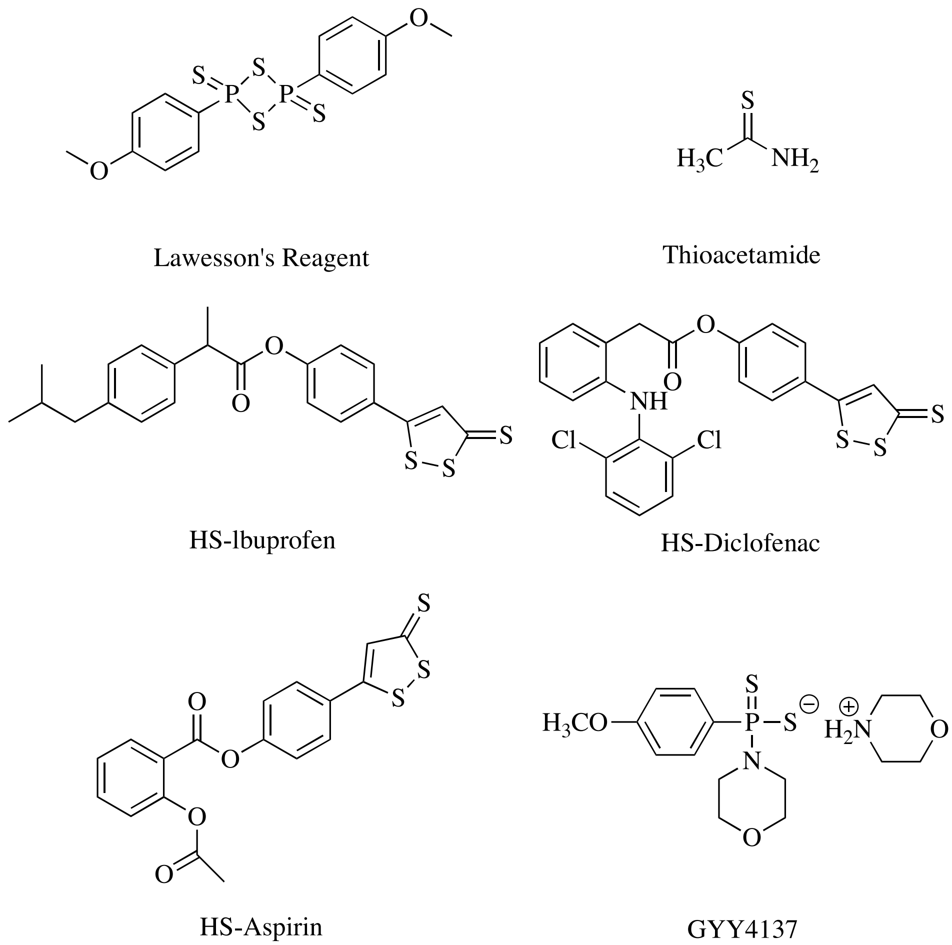
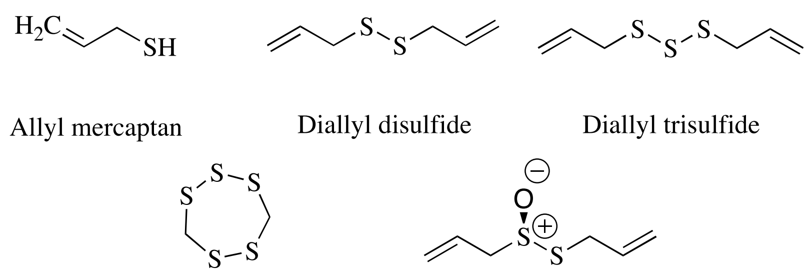


Figure 2. Select H_2S generating compounds.

Another exogenous source of sulfide is sulfur-containing phytochemicals. These are commonly found in foods such as garlic or onions, and include allyl mercapton, diallyl disulfide, diallyl trisulfide, lenthionine, and allicin (Figure 3).[2,16,21]



Allyl mercaptan Diallyl disulfide Diallyl trisulfide

Lenthionine Allicin

Figure 3. Selected phytochemical sources of H_2S.

1.3 Removal of H_2S from the Body

Several pathways in the body metabolize hydrogen sulfide. [10,22] One common pathway is the oxidation of H_2S by various enzymes, followed by excretion of the by-products in the urine.[10] Sulfide is converted to sulfite (SO_3^{2-}) and is then oxidized to thiosulfate ($S_2O_3^{2-}$) and finally sulfate (SO_4^{2-}), which is removed by the kidney.[4,10,14] Another metabolic pathways in the removal of H_2S includes 'scavenging.'[4] H_2S could be bound to metallo- or disulfide- containing molecules, such as proteins that contain heme moieties or cysteine.[4] The toxicity of H_2S is partially caused by binding to the iron in mitochondrial chytochrome, preventing cellular respiration; however, it is possible that the binding of H_2S to the iron in hemeoglobin could also be a "sink" for the three gasotransmitters, CO, NO, and H_2S.[4]

1.4 Biological Effects of H_2S

Although H_2S is more commonly known as a toxic compound, it has recently emerged as a cell-signaling agent.[12,14,23-30] H_2S has been implicated in many biological process including vasorelaxation, neuromodulation, regulation of ion channels, inflammation, apoptosis, cytoprotection, myocardial contractility, vascular tone, and blood pressure.[14,31-36] There have been conflicting papers published on several of the biological effects of H_2S, including the observation of both pro- and anti-inflammatory effects.[37] This is possibly due to several reasons, including concentration of H_2S or the selectivity of inhibitors of H_2S synthesizing enzymes.[10,37] Wallace et al. reviewed 20 papers utilizing the inhibitor PAG to examine the biological effects of H_2S; however, only the L-isomer is an inhibitor of CSE while the R-isomer is toxic.[37] Seventeen of the 20 papers Wallace et al. looked at used the racemate D/L-PAG, causing concern about the conclusions drawn from the inhibition of H_2S production.[37] As with many compounds, the difference between a medicine and a poison is in the dose; in this case it is possible that conflicting reports are unknowingly using different concentrations of H_2S.[37]

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Chapter 2

Generation of Reactive Oxygen Species via the Autoxidation of H_2S under Physiologically Relevant Conditions

2.1 Introduction

Hydrogen sulfide (H_2S) has long been known for its toxic properties[1-5]; however, in recent years, evidence has emerged that this small molecule may serve as a cell signaling agent in mammals.[6-13] H_2S has been implicated in the modulation of diverse processes including inflammation,[14] angiogenesis,[15] cytoprotection,[16] nociception,[17] stimulation of ATP-sensitive potassium ion channels,[18] myocardial contractility,[19] and vascular tone and blood pressure.[20-21] Some sulfur-containing small molecules including leinamycin,[22] 1,2-dithiolan-3-ones,[23] polysulfides,[22,24-26] varacin,[22,24-26] lissoclinotoxin A,[22,24-26] 3*H*-1,2-dithiole-3-thiones,[27-28] and garlic-derived phytochemicals such as *S*-allylcysteine,[29-30] allicin,[29-30] diallyl disulfide,[20,31] and diallyl trisulfide[20] (Figure 1) may gain at least a portion of their bioactivities via the release of H_2S.

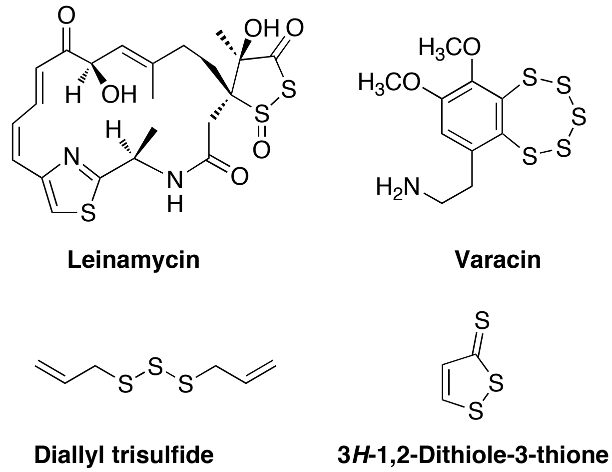


Figure 1. Various sulfur containing molecules.

Though perhaps surprising in light of its potential role as an endogenous signaling agent, there is evidence that H_2S may be a DNA-damaging mutagen. For example, H_2S showed genotoxicity in a modified comet assay where DNA repair was inhibited[32] and in nontransformed human intestinal epithelial cells.[33] In naked nuclei of Chinese hamster ovary cells, H_2S caused nucleobase damage that was excised by the repair enzyme formamidopyrimidine glycosylase (FPG).[34] Evidence for the oxidative nature of this base damage was inferred from the observation that the radical scavenger *t*-butylhydroxyanisole inhibited its formation. In a separate study, H_2S was found to cause increased levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine in coelomocytes and in *Glycera dibranchiata*.[35] In cultured human lung fibroblasts, H_2S induced a concentration-dependent increase in micronuclei, a finding suggestive of DNA damage.[36] Finally, H_2S was shown to be weakly mutagenic in the *Salmonella typhimurium* strain 1535.[37] Transition metal-dependent autoxidation of H_2S in the environment has been characterized,[38-39] but the generation of DNA-damaging reactive oxygen species by these processes under physiologically-relevant conditions has not been well studied. In the work reported here, the chemical mechanisms of DNA damage initiated by the autoxidation of H_2S under physiological conditions were examined.

2.2 DNA strand cleavage by H_2S

H_2S can be introduced into aqueous solutions as the gaseous form or as the salts Na_2S and NaSH.[40] Regardless of the form in which it is introduced into solution, at pH 7.4 and 25 ˚C, an equilibrium mixture composed of H_2S (~30%) and the monoanion HS^- (~70%) is established, with the dianion S^{2-} present in a very small amount (for H_2S, pK_{a1} = 6.98, pK_{a2} ~ 19).[40] Here, we refer to this collective equilibrium mixture as "H_2S". Sulfur anions readily undergo trace metal-mediated oxidation in aerobic solution to generate superoxide radical ($O_2^{\bullet-}$, Eqns 1-3).[26,38,41-45] Superoxide radical disproportionates to yield hydrogen peroxide (H_2O_2) that, in turn, can undergo a Fenton-type reaction involving adventitious traces of transition metals to yield the well known DNA strand cleaving agent hydroxyl radical (HO•, Eqns 4 and 5).[46] Accordingly, we examined the activity of H_2S in a plasmid-based assay that readily measures strand cleavage by reactive oxygen species (ROS).[22,24,26] In this assay, single-strand cleavage converts supercoiled plasmid DNA (form I) to the open-circular form (form II).[47-49] The two forms of plasmid DNA are then separated using agarose gel electrophoresis, the gel is stained with a DNA-binding dye such as ethidium bromide, and the relative amounts of cleaved and intact plasmid are quantitatively determined by digital image analysis. Direct strand breaks (not requiring thermal or basic workup) monitored in this type of experiment typically arise via the reaction of radicals with hydrogen atoms on 2'-deoxyribose residues in the backbone of DNA.[50-53]

Using Na_2S as a source of H_2S we observed concentration-dependent cleavage of duplex DNA (Figure 2). At Na_2S concentrations in the range of 10-1000 μM, nicked (form II) plasmid resulting from single-strand cleavage was observed, while no linearized (form III) plasmid arising from double-strand breakage was seen. Significant strand cleavage was observed at Na_2S concentrations as low as 10 μM. The data suggests a non-linear increase in the yields of DNA strand breaks with increasing Na_2S concentration in the range 10-1000 μM (Figure 2B). Previous reports indicate that high $H_2S{:}O_2$ ratios favor the formation of elemental sulfur as an oxidation product.[42,54] Elemental sulfur may react with HS^- to generate polysulfides S_n^{2-} that, in turn, react with O_2 to generate additional superoxide radical.[39,42,54-55] To probe this possibility directly, we investigated the effect of added elemental sulfur (S_8, added as a suspension in water) on DNA cleavage by Na_2S. These reactions were carried out under our standard reaction conditions reported in the Legend of Figure 2, except at 24 °C. S_8 alone (16 nM) generated 0.44 ± 0.20 strand breaks above background, comparable to the 0.41 ± 0.11 strand breaks generated by Na_2S (250 μM) alone. The combination of S_8 (16 nM) and Na_2S gave a synergistic increase to yield 1.98 ± 0.10 strand breaks above background.

Figure 2A Figure 2B

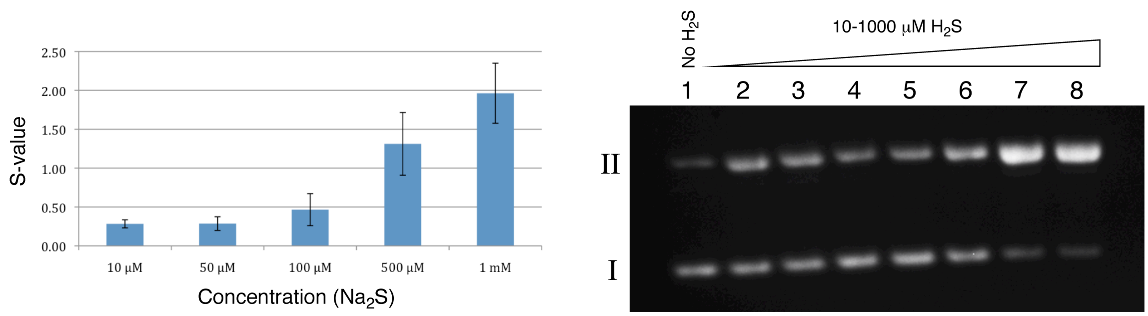


Figure 2. DNA strand cleavage by H_2S. Strand cleavage assays were performed as described in the Experimental Section. Briefly, supercoiled double-stranded plasmid DNA (pGL2-Basic, 1 µL of a 1 mg/mL solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to water (17 µL), followed by addition of sodium sulfide nonahydrate (2 µL in sodium phosphate, pH 7, 500 mM). After 12 h incubation, Form I and II plasmid DNA were resolved on an agarose gel and stained with ethidium bromide. The DNA was visualized by UV-transillumination and the amounts measured by digital image analysis. The number of single strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_I$ where f_I is the fraction of plasmid present as form I.[56] **Panel A.** A plot of strand cleavage yields (S) versus H_2S concentration. Here the yields of strand cleavage were corrected for the amount of form II plasmid present in untreated plasmid DNA (lane 1). **Panel B.** Treatment of plasmid DNA with H_2S led to an increase in the amount of cleaved, form II DNA. Lane 1 contained plasmid with no H_2S while lanes 2-8 contained 10, 25, 50, 100, 250, 500, and 1000 µM H_2S, respectively.

2.3 Mechanism of DNA strand cleavage by H_2S

As noted above, trace metal-mediated autoxidation of HS^- may generate $O_2^{\bullet-}$.[26,38,41-45] To probe the involvement of $O_2^{\bullet-}$, H_2O_2, HO•, and adventitious transition metals in strand cleavage by H_2S, we performed a series of cleavage assays in the presence of additives that interact with various species shown in Scheme 1, Eqns 2-5.[46] For example, we found that strand cleavage was inhibited by the classical hydroxyl radical scavengers[46] methanol, ethanol, and DMSO (Table 1). The hydrogen peroxide-destroying enzyme catalase also inhibited strand cleavage.

Table 1. Effect of additives on DNA cleavage by H_2S.[a]

Reaction/Additive	% Nicked, Form II DNA	S-value[b]
DNA Alone	32.2	0.39±0.04
250 μM Na_2S (Std.)	49.8	0.70±0.09
Std. + methanol (500 mM)	33.1	0.40±0.06
Std. + ethanol (500 mM)	31.4	0.38±0.05
Std. + mannitol (100 mM)	32.8	0.40±0.04
Std. + DETAPAC (10 mM)	29.8	0.36±0.03
Std. + SOD (100 μg/mL)	88.2	2.18±0.23
Std. + catalase (100 μg/mL)	42.4	0.55±0.03

[a] Strand cleavage assays were performed as described in the Experimental Section. Briefly, supercoiled double-stranded plasmid DNA (pGL2-Basic, 1 μL of a 1 mg/mL solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to water (17 μL), followed by addition of sodium sulfide nonahydrate (2 μL in sodium phosphate, pH 7, 500 mM). Form I and II plasmid DNA were resolved on an agarose gel and stained with ethidium bromide. The DNA was visualized by UV-transillumination and the amounts measured by digital image analysis. [b] The number of single strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$ where f_1 is the fraction of plasmid present as form I.[56]

Interestingly, addition of superoxide dismutase (SOD) significantly *increased* the yield of DNA strand breaks. There are at least two possible reasons for this effect, both of which are consistent with the reaction cascade shown in Scheme 1, Eqns 1-5. First, SOD catalyzes the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2.[46] Although spontaneous disproportionation of $O_2^{\bullet-}$ is fast,[46,57] the ability of SOD to accelerate this reaction has the potential to increase the yield of H_2O_2 formation, thus increasing the yields of strand cleavage stemming from the reactions shown in Scheme 1, Eqns 1-5. Second, and likely more important, SOD acts as an HS^-:O_2 oxidoreductase that converts HS^- and O_2 into H_2O_2 and S˚ (Scheme 1, Eqn 6), where S˚ is defined as elemental sulfur and related "sulfane" species in which sulfur is bonded only to sulfur).[58] Under the reaction conditions employed here, the resulting elemental sulfur is expected[25,59] to react with HS^- to generate polysulfides (S_n^{2-}) that can react with O_2 to generate additional superoxide radical via reactions analogous to those shown in Scheme 1, Eqns 2 and 3. Indeed, we presented evidence above showing that addition of elemental sulfur to a standard Na_2S reaction significantly increased strand cleavage.

$$H_2S \rightleftharpoons HS^- + H^+ \quad (1)$$

$$HS^- + M^{(n+1)+} \rightarrow HS\bullet + M^{n+} \quad (2)$$

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2\bullet^- \quad (3)$$

$$O_2\bullet^- + HO_2\bullet \rightarrow H_2O_2 + O_2 \quad (4)$$

$$H_2O_2 + M^{n+} \rightarrow HO\bullet + HO^- + M^{(n+1)+} \quad (5)$$

$$HS^- + O_2 \xrightarrow{SOD} S^\circ + H_2O_2 \quad (6)$$

Scheme 1: Reactions involved in the autoxidation of H_2S.

DNA cleavage also was effectively suppressed by the presence of diethylenetriaminepentaacetic acid (DETAPAC), a chelator of adventitious metals that inhibits transition metal-dependent Fenton-type reactions (Scheme 1, Eqn 5).[46] In analogy with the ability of chelators to inhibit metal-mediated oxidation of organic thiolates (RS^-),[45] it also was expected that DETAPAC could inhibit the initial metal-mediated oxidation of HS^- (Scheme 1, Eqn 2).[38] To provide further evidence for the role of metals in the H_2S-mediated strand cleavage process, we carried out a complementary experiment in which we added small amounts of the transition metal Fe(II) to the reaction mixtures, which quickly became oxidized to Fe(III) in solution. We found that Fe(II) concentrations between 1 nM and 1 μM significantly increased strand cleavage induced by H_2S (Table 2). Fe(II) at these low concentrations did not induce significant strand cleavage on its own. These results confirmed the metal-dependence of H_2S-mediated strand cleavage processes.

Table 2. Cleavage of plasmid DNA by H_2S in the presence of various concentrations of iron.[a]

Reaction/Additive	% Nicked, Form II DNA	S-value[b]
DNA Alone	33.9	0.40±0.05
250 μM Na_2S alone (Std.)	48.2	0.60±0.04
Std. + $FeSO_4$ (1 nM)	53.7	0.80±0.14
Std. + $FeSO_4$ (100 nM)	66.3	1.01±0.06
Std. + $FeSO_4$ (1 μM)	83.9	1.83±0.04
$FeSO_4$ alone (1 μM)	32.1	0.36±0.07

[a] Strand cleavage assays were performed as described in the Experimental Section. Briefly, supercoiled double-stranded plasmid DNA (pGL2-Basic, 1 μL of a 1 mg/mL solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to water (17 μL), followed by addition of sodium sulfide nonahydrate (2 μL in sodium phosphate, pH 7, 500 mM). Form I and II plasmid DNA were resolved on an agarose gel and stained with ethidium bromide. The DNA was visualized by UV-transillumination and the amounts measured by digital image analysis. [b] The number of single strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$ where f_1 is the fraction of plasmid present as form I.[56]

2.4 Investigating strand cleavage by the H_2S decomposition products thiosulfate, sulfite, and sulfate

H_2S readily undergoes oxidation both in aerobic solution and in cells.[8,40,60-63] The products of this oxidation process include thiosulfate ($S_2O_3^{2-}$), sulfite (SO_3^{2-}), and sulfate (SO_4^{2-}).[8,40,64] Given that sulfite and bisulfite (HSO_3^-, the protonated form of sulfite), at least, have been reported to undergo metal-mediated autoxidation reactions that generate reactive oxygen and sulfur species,[65-67] we felt it was important to investigate the ability of various H_2S oxidation products to cause strand cleavage under the conditions of our assay. In the event, we found that none of these H_2S oxidation products generated significant levels of strand cleavage. Overall, the data indicates that the expected H_2S oxidation products thiosulfate, sulfite, and sulfate do not contribute to the DNA strand cleavage observed under our reaction conditions.

Table 3. Cleavage of plasmid DNA by H_2S oxidation products.[a]

Reaction/Additive	% Nicked, Form II DNA	S-value[b]
DNA Alone	31.8	0.33±0.02
500 μM Na_2S	43.2	0.56±0.08
1 mM Na_2S	46.3	0.63±0.12
500 μM NaS_2O_3	27.8	0.30±0.02
1 mM NaS_2O_3	28.1	0.30±0.02
500 μM Na_2SO_3	28.3	0.31±0.04
1 mM Na_2SO_3	27.8	0.29±0.03
500 μM Na_2SO_4	23.7	0.23±0.02
1 mM Na_2SO_4	24.7	0.23±0.04
500 μM $NaHSO_3$	29.8	0.28±0.00
1 mM $NaHSO_3$	31.4	0.31±0.03

[a] Strand cleavage assays were performed as described in the Experimental Section. Briefly, supercoiled double-stranded plasmid DNA (pGL2-Basic, 1 μL of a 1 mg/mL solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to water (17 μL), followed by addition of sodium sulfide nonahydrate (2 μL in sodium phosphate, pH 7, 500 mM). Form I and II plasmid DNA were resolved on an agarose gel and stained with ethidium bromide. The DNA was visualized by UV-transillumination and the amounts measured by digital image analysis. [b] The number of single strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$ where f_1 is the fraction of plasmid present as form I.[56]

2.5 Effect of thiols on DNA strand cleavage by H_2S

Cells contain millimolar concentrations of thiols such as glutathione.[68-70] Therefore, we examined the effects of added thiols on the ability of H_2S to cause DNA strand cleavage. We find that Na_2S (1 mM) in the presence of 2-mercaptoethanol (10 mM) yields 1.2 ± 0.3 strand breaks above background. Under the same conditions – except in the absence of thiol – Na_2S generates 1.9 ± 0.4 strand breaks above background. The compound 2-mercaptoethanol alone (10 mM) generates 0.6 ± 0.5 strand breaks above the background levels of strand breaks present in the plasmid substrate, under the reaction conditions employed here. The result of this control reaction is consistent with previous reports indicating that thiols alone generate DNA strand breaks via autoxidation processes that produce ROS.[71-72] Overall, our results provide evidence that H_2S generates DNA strand cleavage in the presence of thiols, although the cleavage yields are somewhat diminished. Similar results were observed at lower concentrations of Na_2S and thiol and with the biological thiol, glutathione (Table 4). In general, thiols have the potential to act as either prooxidants or antioxidants. When incubated alone in the plasmid-based DNA-cleavage assay, the mild prooxidant properties of 2-mercaptoethanol are displayed. However, with respect to the strand cleavage caused by Na_2S added thiol appears to serve as an antioxidant.

Table 4. Cleavage of plasmid DNA by H_2S in the presence of various thiols.[a]

Reaction/Additive	% Nicked, Form II DNA	S-value[b]
DNA Alone	32.7	0.40±0.01
250 μM Na_2S (Std.)	51.8	0.74±0.08
Std. + GSH (1 mM)	71.7	1.44±0.46
Std. + GSH (5 mM)	96.7	3.41
Std. + cys (1 mM)	56.5	0.84±0.09
Std. + cys (5 mM)	73.5	1.80±0.84
Std. + DTT (1 mM)	54.5	0.81±0.15
Std. + DTT (5 mM)	53.2	0.77±0.08
GSH alone (5 mM)	60.6	1.12±0.49
Cys alone (5 mM)	59.7	1.22±0.64
DTT alone (5 mM)	85.2	1.93±0.14

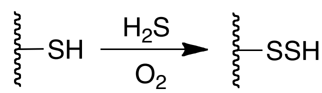
aStrand cleavage assays were performed as described in the Experimental Section. Briefly, supercoiled double-stranded plasmid DNA (pGL2-Basic, 1 μL of a 1 mg/mL solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to water (17 μL), followed by addition of sodium sulfide nonahydrate (2 μL in sodium phosphate, pH 7, 500 mM). Form I and II plasmid DNA were resolved on an agarose gel and stained with ethidium bromide. The DNA was visualized by UV-transillumination and the amounts measured by digital image analysis. *b* The number of single strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$ where f_1 is the fraction of plasmid present as form I.[56]

2.6 Discussion

Our results indicate that H_2S undergoes trace metal-mediated autoxidation to generate superoxide, hydrogen peroxide and, ultimately, the well-known DNA-cleaving agent hydroxyl radical. The metal dependence of this process is consistent with previous reports regarding the role of transition metals in the environmental oxidation of sulfide.[38-39] Though cells contain little or no free transition metals, it is clear that protein bound metals are capable of participating in such redox processes.[46] Indeed hemeprotein-mediated autoxidation processes have been suggested to contribute to the cytotoxicity of H_2S.[3] It may be significant that, at physiological pH, significant amounts of H_2S exist as the sulfur anion HS^- that is the principal substrate for aerobic oxidation.[40,44] In contrast, typical pK_a values for thiols are substantially higher (e.g. the pK_a of the thiol group in cysteine is 8.3) and relatively small amounts of the thiolate anions (RS^-) are present.

Our results showed that the H_2S oxidation products thiosulfate, sulfite, and sulfate do not contribute to the strand cleavage processes examined here. On the other hand, initially-generated oxidation products such as persulfide (S_2^{2-}) likely undergo rapid autoxidation reactions that contribute to the generation of superoxide under our reaction conditions.[39,42,54-55] The non-linear increase in DNA strand cleavage with increasing H_2S concentrations may mesh with previous reports indicating that high H_2S:O_2 ratios favor the formation of elemental sulfur as an oxidation product.[42,54] Elemental sulfur may react with HS^- to generate polysulfides S_n^{2-} that, in turn, react with O_2 to generate additional superoxide radical.[39,42,54-55] In this manner, polysulfides act as catalysts for sulfide oxidation and the concomitant production of ROS.[22,73] Indeed, we provided evidence here that addition of even small amounts of elemental sulfur dramatically increased the DNA-cleaving properties of Na_2S. Finally, we showed that DNA strand cleavage by H_2S proceeded in the presence of physiological thiol concentrations.

Redox chemistry of the type described here could underlie much of the biological activity associated with H_2S. For example, the ability of H_2S to generate superoxide, hydrogen peroxide, and hydroxyl radical under physiological conditions may explain the mutagenic properties of H_2S.[32,34-37] The hydroxyl radical is a well characterized mutagen.[52,74] Furthermore, H_2O_2 produced in the autoxidation of H_2S may be relevant to the putative cell signaling properties of H_2S, given that H_2O_2 has recently become established as a cell signaling agent in its own right.[75-81] Thus, it is possible that, under some circumstances, H_2S serves as a means for generating H_2O_2 in cell signaling processes. Our results further highlight a potential role for the enzyme superoxide dismutase in catalyzing the generation of H_2O_2 from H_2S.[58] The production of ROS (specifically H_2O_2) may explain the ability of H_2S to activate the transcription factors such as Nrf2.[16,82] Likewise, H_2S could contribute to the activation of Nrf2 by agents such as 3*H*-1,2-dithiole-3-thiones and diallyl sulfides.[20,83-88] The oxidation of H_2S in the presence of protein thiols has the potential to generate protein persulfides (Scheme 2). It has been suggested that such protein sulfhydration reactions are involved in the cell signaling properties of H_2S.[10,89-90] Finally, it has been proposed that sulfane byproducts of H_2S oxidation (e.g. S_n^{2-}, S_8) may be the actual regulatory agents generated by H_2S.[91-92]



Scheme 2: Generation of protein persulfides from thiols via the oxidation of H_2S.

Our work highlights some similarities between the cell signaling agents nitric oxide, H_2O_2, and H_2S. Each of these species can mediate controlled biological responses via the selective reactions with specific target proteins, yet also have the potential to cause toxicity via "off target" reactions with bystander proteins and nucleic acids.[79,93-94] The DNA-damaging properties of H_2S discussed here and elsewhere[32-37] emphasize the importance of spatial control in the generation of H_2S if this agent does indeed serve as a cell signaling agent.

2.7 Materials and Methods

2.7.1 Chemicals and Reagents

Reagents were purchased from the following suppliers and were of the highest purity available: sodium phosphate, ethidium bromide, mannitol, 2-mercaptoethanol, *L*-cysteine, dithiothreitol, and sodium bisulfite from Aldrich Chemical Co.; sodium sulfide nonahydrate, NaSH•xH_2O, and H_2S gas, superoxide dismutase (SOD), catalase, glutathione, sodium thiosulfate pentahydrate, Tris-HCl, EDTA from Sigma Chemical Co.; water (HPLC grade), sodium sulfite, sodium sulfate from Fisher Scientific; absolute ethanol from Decon Labs; diethylenetriaminepentaacetic acid (DETAPAC) from Fluka; and agarose from Lonza. Before use, the sodium sulfide was rinsed with distilled, deionized water to remove oxide impurities from the surface and then dried as described previously (weighing of freshly washed, damp material may lead to a slight underestimation of stock concentrations).[95]

2.7.2 Cleavage of Plasmid DNA by H_2S

CAUTION: H_2S is highly toxic. Exposure to this gas can be fatal. Appropriate precautions must be taken when working with H_2S gas and aqueous solutions containing the salts Na_2S and NaSH.[40] In a typical assay, supercoiled double-stranded plasmid DNA (pGL2-Basic, 1 μL of a 1 mg/mL solution in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to water (17 μL), followed by addition of sodium sulfide nonahydrate (Na_2S, 2 μL in sodium phosphate, pH 7, 500 mM). The solution was gently mixed, spun for 2 s in a tabletop centrifuge, and incubated at 37 °C for 12-14 h. Solutions of sodium sulfide were prepared immediately before use and used within 5 min of preparation. In mechanistic experiments where various additives were present in the assays, these agents were placed in the reaction mixture prior to the addition of DNA. After incubation, loading buffer (5 μL of a 50% glycerol loading buffer[96]) was added and the reaction mixtures were loaded onto a 0.9% agarose gel. The gel was electrophoresed for 2-3 h at 80 V in 1x TAE buffer and then stained in a dilute solution of aqueous ethidium bromide. The gel was placed on a UV transilluminator and the amount of DNA in each band quantified using an Alpha Innotech IS-1000 digital imaging system. Concentrations of NaSH solutions were calculated using a molecular weight of 56.1 g/mol. Because the NaSH salt contains waters of hydration (usually about 2-3), the reported concentrations are somewhat higher than the actual concentrations.

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