HYDROGEN SULFIDE AND ITS POTENTIAL ROLE AS AN OXYGEN SENSOR

A Dissertation

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by

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Hydrogen sulfide has in the last 15 years been elevated from its previous place as nothing more than a toxic gas to the status of ubiquitous gasotransmitter with numerous physiologic functions. In this thesis the focus is two-fold: whether H₂S functions as a gasotransmitter in blood and whether H₂S could act more locally as an endogenous oxygen sensor.

The initial research presented here examines sulfide measurement techniques for biologic samples, and assessed their applicability for use in blood and plasma. It also shows the development of an amperometric means of conducting this research. It was found that the standard protocol for potentiometric measurement of sulfide in plasma is unsuitable for such use. The experimental conditions liberate sulfide from cysteines in plasma proteins, creating artificially high sulfide measurements. Using the methylene blue method we were unable to replicate blood sulfide measurements found in other studies, while showing unequivocally that this method should work in plasma if sulfide is present. The amperometric sensor developed here may be used in real time without altering the sample. Using this method we showed in blood from numerous animals that free H₂S is undetectable in blood and that blood consumes sulfide quite rapidly.

The second portion of this research showed that tissues consume endogenously-produced H₂S oxygen-dependently and that the likely location of this consumption is in
the mitochondria. The inhibitory level of oxygen was quite low, and the levels of
cysteine required to stimulate measurable production were supraphysiologic, thus an
attempt was made to develop a means of measuring intracellular sulfide. This was done
through sulfide-sensitive Photonic Explorers for Bioanalysis with Biologically Localized
Embedding (PEBBLE). PEBBLEs were produced in an organically-modified silica
matrix using the sulfide-sensitive fluorescent dye fluorescein mercuric acetate. While the
PEBBLEs were sensitive to H$_2$S and insensitive to cysteine, intracellular production of
sulfide was not successfully demonstrated.

H$_2$S may function as an oxygen sensor intracellularly, but it does not circulate in
the blood and is unlikely to exist outside of localized pockets within cells due to rapid
oxidation. Further work should be done to establish a means of intracellular
measurement of H$_2$S.
DEDICATION

This of course must be dedicated to my fiancé Claire, who has been with me through almost all of this, and who wasn’t even my fiancé until I was nearly finished. She brought me dinner too many times to count and has had a superhuman understanding of the endless long nights in the lab it took to get to this point. And also to my parents, whose indulgence of my endless curiosity as a kid set me up for research down the road.
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INTRODUCTION

I. Overview:

Until 1996, when Abe and Kimura reported that hydrogen sulfide may be an endogenous neuromodulator (Abe & Kimura, 1996), H₂S was viewed simply as a toxic gas. A review of H₂S toxicity (Reiffenstein et al., 1992) mentions the wide variety of effects H₂S can have on numerous body systems. It was long known that tissues produced H₂S during normal metabolism, but this production was regarded as simply a byproduct. Since Abe and Kimura’s paper, there has been an explosion of studies, which have reported a myriad of physiological actions of H₂S (discussed below, and see Appendix II), many of which overlap with the toxic effects mentioned by Reiffenstein, and H₂S has risen to the status of a blood-borne gasotransmitter in league with CO and NO.

Inherent in the designation ‘physiological’ is the assumption that the levels of H₂S used experimentally reflect the situation in vivo. Likewise, the designation blood-borne gasotransmitter obviously requires that H₂S be transported in the blood in a form that is usable by downstream tissues. These assumptions are supported in the literature by measurements of hydrogen sulfide in blood, plasma, and tissue, and measurement of hydrogen sulfide production in tissues. Unfortunately the methods typically used are not ideal and do not provide accurate assessments in biological systems, as will be shown in
this dissertation. This does not, however, mean that H₂S does not function locally, and
evidence will also be presented here showing that H₂S may act in an oxygen-dependent
manner, functioning as an oxygen sensor. Lastly, progress will be reported on the
development of an H₂S nanosensor that may in the future allow detection of intracellular
sulfide.

II. Literature Review

II.A. Hydrogen Sulfide Chemistry

Hydrogen sulfide is a highly reactive, flammable, colorless gas. It is the sulfur
analog of water, but is somewhat less polar, lending it the ability to dissolve well in both
polar and non-polar environments. The gaseous nature and lipophilicity of H₂S allows it
to dissolve in cell membranes and thus no transporters are needed for it to enter or leave
the cell (Mathai et al., 2009). H₂S is also a weak acid, and in aqueous solution
dissociates according to Equation 1:

\[
\text{H}_2\text{S} \rightleftharpoons \text{HS}^- + \text{H}^+ \rightleftharpoons \text{S}^{2-} + 2\text{H}^+ \quad (1)
\]

This allows an even larger quantity of sulfide to be dissolved. The pKₐ₁ commonly used
in the literature is 7.05, which at pH 7.4 gives a distribution of ~30% H₂S and 70% HS⁻
(Lide, 1998). This pKₐ is in pure water at 20°C, however, and is thus not accurate for
physiological situations. More accurate values, in saline, are 6.6 at 14°C and 6.9 at 37°C
(Hershey et al., 1988). This then gives a distribution at 37°C of 14% H₂S and 86% HS⁻.
Estimates for the second dissociation constant span a few orders of magnitude, but in all cases they are at least 12. The true value is more likely ~17 (Migdisov et al., 2002), but in any case [S$^2$-] is negligible under physiologic conditions.

H$_2$S is a strong reductant, and can spontaneously oxidize to sulfur dioxide or elemental sulfur (among other possibilities) as shown in Equations 2 and 3, respectively:

\[ 2\text{H}_2\text{S} + 3\text{O}_2 \rightleftharpoons 2\text{H}_2\text{O} + 2\text{SO}_2 \]  \hspace{1cm} (2)

\[ 2\text{H}_2\text{S} + \text{O}_2 \rightleftharpoons 2\text{H}_2\text{O} + 2\text{S}^0 \]  \hspace{1cm} (3)

These reactions are not particularly fast in the absence of catalysts, with half-times on the order of 30 hr (Millero et al., 1987). In the presence of transition metal ions, such as Fe$^{2+}$, the oxidation rate is greatly increased, and addition of the transition metal chelator diethylenetriaminepentacetic acid to an H$_2$S solution effectively blocks this route of oxidation (Tapley et al., 1999). We see evidence of spontaneous sulfide oxidation in our own lab as a yellowing of Na$_2$S stock solutions left overnight.

II.B. Hydrogen Sulfide Measurement Techniques

There is a very wide range of analytical techniques available for detecting sulfide, covering almost all major classes of analytical chemistry. I will briefly mention many of them below, and provide more detailed reviews of techniques commonly used in the physiological literature.
i. Colorimetric

In the current physiological literature, the methylene blue method is the most commonly used analytical procedure for sulfide, as it has a reasonably low limit of detection (micromolar), is very cheap, and is very simple. In this method, a sample is mixed with acidic solutions of N,N-dimethyl-p-phenylenediamine (diamine) and FeCl₃. The reaction of sulfide with the diamine produces methylene blue, which can then be detected colorimetrically, spectrophotometrically, or by other means. According to Lawrence et al., Emil Fischer first described the formation of methylene blue from sulfide in 1886 (Lawrence et al., 2000). Almy adapted Fischer’s method for use on food, using a colorimeter to compare experimental to standard samples and achieving a lower limit of detection of ~1 µM (Almy, 1925). The particularly important development in Almy’s paper was the trapping of liberated H₂S by bubbling it through a solution of zinc acetate. The separation step eliminates interference from other tissue constituents while the formation of ZnS stabilizes the sulfide and prevents both its outgassing and oxidation. The subsequent acidification of the ZnS solution with the assay reagents redissolves the ZnS. Fogo and Popowsky (Fogo & Popowsky, 1949) further optimized Almy’s method and used a spectrophotometer for detection, achieving a lower detection limit of ~0.7 µM. To my knowledge this detection limit has not improved in the 60 years since. Siegel (Siegel, 1965) used Fogo and Popowsky’s spectrophotometric detection method, but increased the assay reagent concentrations to allow the assay to be performed in the sample solution (which I refer to as the ‘direct’ method in the future), as was the case in the original methylene blue method. Siegel demonstrated that numerous sulphydryl-
containing compounds, including 1 mM cysteine, did not interfere, although 1 mM glutathione and 10 mM mercaptoethanol did increase the absorbance somewhat while bisulfite and thiosulfate strongly inhibited color formation. It was also shown that phosphate, Tris, acetate, and ethylenediaminetetraacetic acid (EDTA had negligible effects, while 1 mM pyridoxal phosphate (commonly used in H₂S production studies) decreased absorbance by ~10%. Siegel reported a detection limit of ~2 µM, which is in line with my experience, and his method is the basis for the majority of sulfide measurements in recent physiological reports.

Stipanuk and Beck (Stipanuk & Beck, 1982) modified Fogo and Popowsky’s trapping method by adding the zinc acetate solution to a piece of filter paper placed inside the sample container. After the sample is acidified with trichloroacetic acid (typically after the sample has had time to enzymatically produce sulfide) and incubated for an hour, the zinc acetate-soaked filter paper is removed and assayed for sulfide as per Siegel. This procedure is the basis for nearly all recent reports of H₂S production in physiological studies, and is what I refer to as the ‘indirect method’. Although both Siegel’s and Stipanuk and Beck’s methods are used ubiquitously for measuring H₂S in blood or plasma, I have been unable to find any reference that rigorously establishes their suitability for such use (e.g. using standard additions and/or titrating stock solutions to verify their sulfide content).
ii. Potentiometric

Potentiometry is an electrochemical analytical technique that relies on the measurement of the potential difference between a working electrode, which responds to the analyte, and a reference electrode, which does not. In the combination silver/sulfide ion-selective electrode (ISE) like the one used in this thesis and many of the papers cited herein, the working electrode is a disc of compressed silver sulfide crystals. The Ag₂S is nearly insoluble in water, with a K\text{sp} of ~10^{-51} (Crombie \textit{et al.}, 1974), and when it is placed in an aqueous sample solution an extremely small amount of the Ag₂S dissolves. The resulting free Ag\textsuperscript{+1} ions are mobile in the sulfide matrix, allowing a potential to develop that is dependent upon the solubility of the Ag₂S. The Ag₂S solubility, in turn, is dependent upon the concentration of sulfide (or silver) in the sample solution, thus allowing determination of the sulfide (or silver) concentration (Skoog \textit{et al.}, 1998). The silver/sulfide ISE does not have many interferences, but it will respond to mercury and cyanide. The reference electrode is contained within the body of the sensor (thus making this a ‘combined’ sensor, although it is still referred to as an ‘electrode’) and is of the Ag/AgCl type, identical to that found in the typical lab combination pH electrode.

An important point about the sulfide ISE is that it responds to S\textsuperscript{2-} and possibly HS\textsuperscript{−}, but not to H\textsubscript{2}S. This necessitates that sample solutions be made very alkaline (pH >10) in order to convert all of the sulfide to S\textsuperscript{2-}. Clearly a pH of 10 is not a physiologic condition, and is alkaline enough to desulfurate cysteines contained in proteins and cause an artificially elevated sulfide measurement (Khan, 1980).
iii. Voltammetric

Voltammetry is an analytical technique in which the voltage of a working electrode is scanned over some voltage range while measuring the resulting current. The measured current is related to the reduction and/or oxidation of species in the analyte solution, which occurs at specific voltages for the different species. If these potentials are different enough, multiple species can be measured with a single electrode in a single voltage scan. Brendel and Luther (Brendel & Luther, 1995) used this technique with a gold-mercury amalgam working electrode to measure sulfide in marine sediments simultaneously with Fe, Mn, and O₂, with a detection limit for sulfide of ~0.2 µM. More recently Lawrence et al. (Lawrence et al., 2004) used 5 µm diameter electrodes covered with carbon nanotubes to voltammetrically measure sulfide, with a detection limit of 0.3 µM. This technique is more technically difficult and requires more specialized equipment than the colorimetric or potentiometric methods.

iv. Amperometric

Amperometry is a subclass of voltammetry in which the current between a working electrode and counter electrode, resulting from some manner of electrochemistry, is measured while keeping the potential between the two electrodes fixed. The H₂S sensor used in the research presented in this dissertation is of the amperometric variety. It is possible to directly oxidize sulfide to sulfur at the working electrode, but the resulting coating of the electrode with deposits rapidly inactivates the electrode. In order to avoid this problem, Jeroschewski et al. (Jeroschewski et al., 1988)
proposed using ferri(o)cyanide as a redox mediator, in which sulfide is oxidized to elemental sulfur by ferricyanide, with the resulting ferrocyanide reoxidized at the anode. The background (baseline) current is from ionic conduction and ferri(o)cyanide redox. The authors first used this system in a galvanic sensor (Jeroschewski et al., 1993) and later proposed an amperometric microsensor, similar to the ubiquitous Clark oxygen sensor, as a practical way to measure H$_2$S in aqueous settings (Jeroschewski et al., 1996). Their design was modified by Doeller et al. (Doeller et al., 2005), whose modification was the basis for my design. Doeller et al. refer to the sensor as polarographic, although polarography technically only applies to a dropping mercury electrode, and the sensor is more accurately termed amperometric.

v. Fluorometric

This method is based on the quenching of fluorescein mercuric acetate (FMA) by sulfide. FMA was first synthesized by White in 1920 (White, 1920), but this was in the context of developing treatments for urinary tract infections and syphilis. The first description I am aware of for FMA’s use in determining sulfide was by Karush et al. (Karush et al., 1964), although they used FMA for determining disulfide bonds and not H$_2$S. In 1965 Andrew and Nichols described a method for using FMA to detect H$_2$S in air, using a counter-current flow of air and FMA solution with an in-line fluorescence detector, and a detection limit of 0.5 parts per billion volume (Andrew & Nichols, 1965). Cardoso et al. (Cardoso et al., 1997) described a method for detection in air that used a hanging drop of FMA solution excited through the solution outlet and detected by a
photodiode adjacent to the drop, with a detection limit of 28 ppbv. Also in 1997, Choi and Hawkins immobilized FMA on ethyl cellulose membranes (overhead transparencies) with tetraoctylammonium hydroxide to create a sulfide-sensitive optode membrane which can be used with a fiber optic detection system, and has a detection limit of 56 nM (Choi & Hawkins, 1997). A field-usable device has been developed which passes a sample gas (i.e. air) over an H$_2$S membrane covering a channel through which FMA solution runs, with quenching measured by a photo diode (Toda et al., 2001). This technique has a wide usable range and a lower limit of detection of 0.1 ppb. Fluorometric techniques inherently have a high sensitivity due to the ease with which low levels of light may be measured, and my work on intracellular detection is based on a fluorometric method.

vi. Titrimetric

Titration is not a particularly sensitive or interference-free measurement technique for sulfide, but it is a critical technique because it does not require prior knowledge of any stock sulfide concentration in order to measure an unknown sulfide concentration. All the other methods mentioned here arrive at sulfide concentrations based on the comparison of samples to a sulfide standard curve, and clearly if the standard concentrations are inaccurate, the experimental concentrations will be inaccurate. Titration enables the standardization of the relatively unstable sulfide stock solutions using more stable compounds whose concentrations can be more accurately and precisely known.
In iodometric titrations, an excess but known amount of iodine is used to quantitatively oxidize the sulfide in a sample, and the remaining iodine is then back-titrated with a standardized thiosulfate or thiocyanate solution to determine the amount of sulfide in the sample. An analogous titration can be accomplished using silver ion instead of iodine, back-titrating with thiocyanate (Kolthoff, 1969).

vii. Lead Acetate Tape

There are many variations of this technique, but the basic principle is that colorless lead acetate on a strip of paper is converted to the dark PbS upon exposure to H₂S, and the paper can then be visually inspected or analyzed by transmittance or reflectance. There is a wide range of automation and sensitivity available with this method (Garber et al., 1970).

viii. Turbidometric

Bismuth sulfide can be precipitated from a sulfide-containing solution by adding bismuth nitrate, and the sulfide quantified in a spectrophotometer using the increase in absorbance caused by the opaque precipitate. This method has a limit of detection of 1.4 µg sulfide. (Field & Oldach, 1946). A second method from Field and Oldach’s paper reacts hydroxide-trapped sulfide with a solution of uranyl nitrate / cadmium acetate, with the resulting color and opacity compared to a standard, with a limit of detection 30 µg.
II.C. Toxicology

Humans have been aware of hydrogen sulfide, or at least its smell, for the entirety of our existence. Hydrogen sulfide gives rotten eggs their distinct smell, as well as lending its smell to flatus and various geologic processes. The first known description of its toxicity dates to 1713 in *De Morbis Artificum Diatriba*, an occupational health manual written by Bernardino Ramazzini (Franco & Franco, 2001). As mentioned earlier, until 1996 most of the research on H₂S focused on its toxicity.

Prior to the advent of industry, the most common sources of environmental H₂S were geochemical processes and anaerobic production in sediment, but H₂S can be produced essentially anywhere anoxia, bacteria, and a source of sulfur (in any number of oxidation states) are found together. There exists precedent for fatalities from natural H₂S, in a case of three ski patrol members who fell into a volcanic fumarole, immediately lost consciousness and died (Cantrell & Young, 2009). Upon the introduction of sewers and animal farming, a new hazard arose from accumulation of H₂S in waste-filled pits. Currently H₂S is produced or used in countless industries, including paper pulp mills, heavy water production, and the natural gas (sour gas) and oil industry (Guidotti, 1996).

The vast majority of H₂S poisonings occur via inhalation, as H₂S easily crosses the alveolar membrane (Evans, 1967). Table 1 is an adaptation from Reiffenstein (Reiffenstein *et al.*, 1992) highlighting the responses in humans to various concentrations of H₂S in air. H₂S may also be absorbed through the skin (Laug & Draize, 1942) and gut (Imamura *et al.*, 1996), but I am not aware of any case reports in which exposure by
either route lead to morbidity or mortality. Prior et. al. (Prior et al., 1988) found in rats an inhaled LC$_{50}$/LC$_{10}$ (lethal concentration at which 50 or 10% die) of 587/549 ppm over 2 hr, 501/422 over 4 hr, and 335/299 over 6 hr. The authors suggest that the very steep dose-response curve (i.e. in the 2 hr experiment a 6% drop in the concentration resulted in an 80% drop in mortality) indicates a detoxification threshold being reached. Almeida et al. (Almeida & Guidotti, 1999) also noticed a steep dose-response curve in intravenous sulfide administration, with an ED$_{0}$ (maximum dose with no deaths) of 0.2 mg kg$^{-1}$ (5.9 µmol kg$^{-1}$) in rats, while the ED$_{100}$ was only 0.6 mg kg$^{-1}$ (17.6 µmol kg$^{-1}$).

### TABLE 1

**HUMAN RESPONSE TO INHALED H$_2$S**

<table>
<thead>
<tr>
<th>Concentration, ppm</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003-0.02</td>
<td>Odor threshold</td>
</tr>
<tr>
<td>3-10</td>
<td>Obvious unpleasant odor</td>
</tr>
<tr>
<td>20-30</td>
<td>Strong offensive odor</td>
</tr>
<tr>
<td>30</td>
<td>Sickening sweet odor</td>
</tr>
<tr>
<td>50</td>
<td>Conjunctival irritation</td>
</tr>
<tr>
<td>50-100</td>
<td>Respiratory irritation</td>
</tr>
<tr>
<td>100-200</td>
<td>Olfactory paralysis</td>
</tr>
<tr>
<td>250-500</td>
<td>Pulmonary edema</td>
</tr>
<tr>
<td>500</td>
<td>Anxiety, headache, ataxia, dizziness, tachypnea, knockdown</td>
</tr>
<tr>
<td>500-1000</td>
<td>Respiratory paralysis, neural paralysis, cardiac arrhythmias, death</td>
</tr>
</tbody>
</table>

Adapted from Reiffenstein et al. (Reiffenstein et al., 1992)
Truong et al. (Truong et al., 2007) reported a mouse LD$_{50}$ of injected NaHS as 0.43 mmol kg$^{-1}$. Laug and Draize reported murine, rabbit, and dog i.v. LD$_{50}$’s for NH$_4$SH of 2 mg kg$^{-1}$, and a murine oral LD$_{50}$ of 80 mg kg$^{-1}$ (Laug & Draize, 1942). The LD$_{50}$ for cats is 25 µmol kg$^{-1}$ i.v. (Evans 1967) and the inhaled lethal dose for rabbits is >500 ppm (Kage 1992).

The response in humans to H$_2$S concentrations above ~500 ppm is essentially instant unconsciousness (termed ‘knockdown’), which can happen after a single breath and will lead to death if the situation is not remedied (Milby & Baselt, 1999). If the individual is quickly removed from the high-H$_2$S environment, however, recovery is typically very rapid and complete. Unfortunately, due to the rapidity of its action, many H$_2$S-related fatalities are actually due to rescue attempts. A study of H$_2$S-related deaths from 1984-1994 found 80 total fatalities, of which 19 were would-be rescuers (Fuller & Suruda, 2000). This rapid death has recently been used, predominantly in Japan, for suicides. Victims mix ‘bath essence’ with HCl-containing toilet bowl cleaner to produce H$_2$S gas, with less than 200 ml each sufficient to create an atmosphere of 1000 ppm in a car (Kobayashi & Fukushima, 2008). In 2008 alone 150 suicides were attributed to this technique, which had been rare only a year earlier (Truscott, 2008). Lower H$_2$S concentrations can also be lethal, but are slower acting. This situation can occur due to the rapid olfactory paralysis that occurs near 200 ppm, preventing the individual from realizing the continuing presence of H$_2$S (Knight & Presnell, 2005).

The traditional explanation for hydrogen sulfide’s toxicity lies in its ability to inhibit cytochrome c oxidase in a manner similar to that of cyanide (Haggard, 1925;
Khan et al., 1990). H$_2$S works more broadly than simple metabolic inhibition, however. An interesting feature of H$_2$S toxicity is the nearly instant apnea upon inhalation of high concentrations. Almeida and Guidotti (Almeida & Guidotti, 1999) demonstrated that venous injection of sulfide caused apnea at a 5x lower dose than carotid injection, and that the vagus nerve was necessary for the lung-induced apnea. The authors suggest that this peripherally-induced apnea could be the primary cause of death in acute poisoning. A second alternative mechanism of apnea is the alteration of neuronal excitability in the central respiratory rhythm-generating centers, also independent of cytochrome c oxidase inhibition (Greer et al., 1995). Any long-term effects are likely not due to the H$_2$S itself but instead to the damage from tissue hypoxia that would result from any type of respiratory arrest (Reiffenstein et al., 1992).

In addition to causing apnea, hydrogen sulfide has a wide range of other reported toxicities. These include olfactory neuron loss in chronic exposure (Brenneman et al., 2000), lung surfactant abnormalities (Green et al., 1991), conjunctival irritation (Evans, 1967), decrease in aspartate, glutamate, and gamma aminobutyric acid in rat pups (Hannah et al., 1989), severe inflammation of rat respiratory surfaces (Lopez et al., 1987), decreased learning in snails (Rosenegger et al., 2004), and increased catecholamine and serotonin levels via inhibition of monoamine oxidase (Warenycia et al., 1989).

A fair amount of research has been done on the forensic identification of H$_2$S-poisoning victims. Nagata et al. (Nagata et al., 1990) examined the blood and organ sulfide levels in rats exposed to lethal concentrations of H$_2$S (550 ppm), and determined
that sulfide was not detectable in control blood, but by four hours after death had increased to the level in exposed rats at the time of death. This led the authors to conclude that blood was not a suitable material for post-mortem sulfide analysis. Brain, lung, and muscle had more stable post-mortem sulfide concentrations, and thus would be appropriate. A subsequent publication by the same authors (Kage, 1992) identified urine and blood thiosulfate as markers of H₂S exposure, but found that that sulfide itself should not be used due to its rapid disappearance from blood. In a later study (Kage et al., 1997) the same authors report the use of both sulfide and thiosulfate to verify death by H₂S inhalation, but indicate that only urine thiosulfate should be used for verifying exposure in non-lethal cases. In a pair of lethal cases reported more recently, however, liver and blood thiosulfate were not found to be elevated (Knight & Presnell, 2005). One possible explanation is that upon exposure to atmospheres sufficiently high in H₂S to cause immediate apnea, the quantity of H₂S inhaled is not enough to elevate thiosulfate levels. Other typical post-mortem findings of severe H₂S exposure include pulmonary edema, scleritis and conjunctivitis, cyanosis, and other signs typical of asphyxiation.

II.D. Physiologic and Pharmacologic Functions of Hydrogen Sulfide

Prior to Abe and Kimura’s report in 1996 (Abe & Kimura, 1996), there were no known physiologic functions for hydrogen sulfide (or hydrosulfide anion; it is not known which is the active species in any given function). Since then many studies have reported functions for endogenously-produced and exogenously-applied hydrogen sulfide. These include neuromodulation (Abe and Kimura, 1996), protection of the gastric mucosa from
oxidative stress (Yonezawa et al., 2007), modulation of leukocyte-mediated inflammation (Zanardo et al., 2006) and leukocyte trafficking (Zhang et al., 2007), inhibition of platelet aggregation (Zagli et al., 2007), inhibition of insulin secretion (Ali et al., 2007), systemic artery vasodilation (Cheng et al., 2004), inhibition of angiotensin converting enzyme (Laggner et al., 2007), aggravation of hemorrhagic shock (Mok et al., 2004), protection from reperfusion injury after myocardial infarction (Sivarajah et al., 2006; Elrod et al., 2007), protection from ambient hypoxia (Blackstone and Roth, 2007), oxygen sensing (Olson et al., 2006) and numerous others (Lowicka and Beltowski, 2007).

The functions mentioned above typically occur at sulfide concentrations between 10 and 300 µM, which are within the range of plasma sulfide levels reported in at least 48 studies since 2000 (see Appendix I). This implies that the above actions are continuously modulated by circulating sulfide. Studies published prior to 2000, however, almost invariably report very low or undetectable levels of plasma sulfide. To my knowledge, in the past nine years only two studies from outside our lab have reported H₂S levels of 5 µM or less, one of which was anecdotal (Koenitzer et al., 2007; Furne et al., 2008). This discrepancy between older and more recent studies does not appear to be due to an improvement in sulfide measurement techniques, as the earlier studies all have detection limits of 1µM or less, and the newer studies, aside from Koenitzer et al., use techniques developed decades ago without any obvious improvements. In fact, in a recent review (Szabó, 2007) the author suggests that circulating H₂S is probably lower than recent reports claim. Throughout the remainder of this literature review there are many instances in which changes in plasma sulfide levels are referenced. Bear in mind that the
data presented in this thesis generally contradict those references in terms of the measured sulfide values, and that while I do not dispute the relative changes reported, I do not believe that the authors’ measurements are in fact measurements of free hydrogen sulfide, but instead of some bound form of sulfide released during the assays.

II.D.i. Endogenous Production of H$_2$S

That hydrogen sulfide can be produced from normal mammalian tissue has been known for at least 70 years (Fromageot et al., 1939; Smythe, 1942). There are multiple vertebrate enzymes that can cleave sulphhydryl groups from their substrates to form H$_2$S (see Figure 1), including 3-mercaptopyruvate sulfurtransferase (3MST), cysteine lyase (CL), cysteine aminotransferase, cystathionine-β-synthase (CBS), and cystathionine-γ-lyase (CSE). The latter two enzymes have received essentially all the attention, although a recent paper reported that 3MST is the most important source of H$_2$S in the brain (Ishigami et al., 2009).

CBS depends, like many amino acid-metabolizing enzymes, on pyridoxal 5’-phosphate (PLP, Vitamin B$_6$) as a cofactor. This enzyme catalyzes a β-replacement reaction with amino acid substrates including cysteine, 3-chloroalanine, and serine. The nucleophile substrates include homocysteine, 2-mercaptoethanol, and H$_2$S. Any combination of the amino acid and nucleophile is possible (Miles & Kraus, 2004). The traditional role of CBS is in the metabolism of homocysteine, in which CBS catalyzes the β-replacement of the sulfhydryl of homocysteine with the hydroxyl of serine, forming
Figure 1. Various pathways for production of hydrogen sulfide in animal tissues. Adapted from Julian et al. (Julian et al., 2002). GCS, 6.3.2.2 = \( \gamma \)-glutamylcysteine synthase; GS, 6.3.2.3 = glutathione synthase; 3MST, 2.8.1.2 = \( \beta \)-mercaptopyruvate sulfurtransferase; CAT, 2.6.1.3 = cysteine aminotransferase; CL, 4.4.1.10 = cysteine lyase; CSE, 4.4.1.1 = cystathionine gamma lyase (inhibited by propargylglycine); CBS, 4.2.1.22 = cystathionine beta synthase (inhibited by aminooxyacetic acid)
cystathionine. Likewise, CBS can catalyze the β-replacement of the sulfhydryl of cysteine with water, releasing serine and H$_2$S (Julian et al., 2002). A second option for producing H$_2$S is the substitution of cysteine’s sulfhydryl with the thiol group of a second compound, releasing a thioether and H$_2$S.

CBS is a highly regulated enzyme. Its C-terminal domain is autoinhibitory and has a binding site for the allosteric regulator S-adenosylmethionine (SAM). When bound, SAM prevents the C-terminal domain from inhibiting CBS’s active site, thus stimulating the enzyme. This serves to adjust the formation of cystathionine to maintain the proper methionine and homocysteine levels (Stipanuk, 2004). CBS also possesses a heme-binding domain toward the N-terminal end. Heme can be considered a cofactor, as it is required for activity, although it does not bind in the active site. The heme also served as a redox regulator, as reducing conditions decrease activity and oxidizing conditions restore activity (Taoka et al., 1998). This could serve to increase production of cysteine, and thus reduced glutathione (GSH), during oxidative stress. In the case of H$_2$S, this could serve a negative feedback function. Cellular redox state has also been shown to affect targeted proteolysis of the SAM-binding domain, thus removing that source of inhibition (Zou & Banerjee, 2003). CBS transcription is also hormonally regulated (Stipanuk, 2004).

The second enzyme typically associated with physiological H$_2$S production is cystathionine γ-lyase (alternately known as cysteine desulphhydrase, which is presumably the source of the standard abbreviation CSE). The reaction for which cystathionine γ-lyase is named is the α,γ-elimination of cystathionine to release α-ketobutyrate and
ammonia, and cysteine. There are numerous reactions by which CSE can directly produce H$_2$S, including $\beta$-replacement of two cysteines to form H$_2$S and lanthionine, $\gamma$-replacement of two homocysteines to form H$_2$S and homolanthionine, $\alpha,\gamma$-elimination of homocysteine and water to form H$_2$S and homoserine, and $\beta$- or $\gamma$-replacement of a homocysteine and cysteine to form H$_2$S and cystathionine. Also, CSE can indirectly lead to H$_2$S production by catalyzing the $\alpha,\beta$-elimination of cystine to produce pyruvate, ammonia, and thiocysteine, which can then spontaneously decompose to cysteine and H$_2$S (Chiku et al., 2009).

The regulation of CSE is not as varied as that of CBS. Yang et al. (Yang et al., 2008) showed that CSE activity is stimulated by Ca$^{2+}$/calmodulin, similarly to endothelial nitric oxide synthase (eNOS) and heme-oxygenase. Li et al. (Li et al., 2005) reported that CSE mRNA was downregulated in high-flow-induced pulmonary hypertension, but do not suggest a mechanism. Beyond these, the only source of regulation I am aware of is simple substrate availability.

A recent addition to the short list of enzymes relevant to physiologic H$_2$S production is 3-mercaptopyruvate sulfurtransferase. 3-mercaptopyruvate is formed from the deamination of cysteine by cysteine aminotransferase. 3MST may cleave mercaptopyruvate to form pyruvate and H$_2$S, or catalyze the transsulfuration of a thiol to a persulfide, which can subsequently join a second thiol to form a disulfide and release H$_2$S. 3MST can be regulated by cellular redox status via its cys$^{247}$. The enzyme is inhibited when the cys$^{247}$ is oxidized to a sulenate (SO$^-$), thus shunting more cysteine to glutathione synthesis (Nagahara et al., 2004). Transcriptional regulation is unlikely, as
the gene for 3MST has the characteristics of a housekeeping gene and it was not induced by numerous potential stimuli (Nagahara et al., 2004).

II.D.ii. Catabolism of Hydrogen Sulfide

As mentioned earlier, H₂S can spontaneously oxidize in the presence of O₂. Even when catalyzed by iron, however, the rate of spontaneous oxidation in the body is small compared to the enzymatic oxidation in mitochondria. While the oxygen-dependent oxidation of sulfide to thiosulfate and/or sulfate as the primary means of sulfide catabolism has been known for most of the past century, the specific steps of mitochondrial oxidation to thiosulfate were only recently described (Hildebrandt & Grieshaber, 2008). The first step uses a membrane-bound sulfide-quinone oxidoreductase (SQR) to oxidize the H₂S to a persulfide (S⁰), with the electrons used to reduce ubiquinone, which then enters the electron transport chain. In the second step a mitochondrial matrix sulfur dioxygenase further oxidizes the persulfide to sulfite, consuming O₂ and water. The sulfite is then converted to thiosulfate by the sulfur transferase-catalyzed transfer of a second persulfide from SQR. Thiosulfate can be excreted by the kidneys or further oxidized to sulfate (Kamoun, 2004).

H₂S can also be removed from the body by simply breathing. It has been shown numerous times that H₂S appears in the breath very shortly after injection of sulfide (Haggard, 1921; Evans, 1967) and thus can cross the alveolar membrane. Practically speaking, however, measurements of H₂S concentrations in exhaled air indicate that very little H₂S is exhaled under normal circumstances (Suarez et al., 1999; Morselli-Labate et
This implies that endogenously-produced H$_2$S is oxidized or bound prior to reaching the lungs, which is in conflict with the findings of the majority of recent papers (see Appendix I and Discussion).

II.D.iii. Neurologic

The first physiologic role proposed for H$_2$S, by Abe and Kimura in 1996 (Abe & Kimura, 1996), was that of a neuromodulator. H$_2$S was reported to enhance n-methyl-D-aspartate (NMDA) receptor-mediated responses which aids in facilitation of long-term potentiation in rat brain. The authors further showed that mRNA for the H$_2$S-producing enzyme CBS was present in rat brain, that brain homogenate produced H$_2$S from cysteine, and that levels at which the neurological effects occurred were within the range of H$_2$S measured in brain tissue (50-160 µM, based on previously published reports). The mechanism was later shown to involve H$_2$S-based stimulation of adenylate cyclase with subsequent activation of protein kinase A, which then activated the NMDA receptors (Kimura, 2000). No mechanism has been proposed to explain the activation of adenylate cyclase, however.

The NMDA activation has been used as a possible explanation for how H$_2$S can increase cerebral ischemic damage (Qu et al., 2006). This hypothesis is based on the observation that both NaHS (Qu et al., 2006)) and cysteine (Wong et al., 2006) increase infarct size in a rat middle cerebral artery occlusion model of stroke. In both cases the CSE/CBS inhibitor aminooxyacetic acid (AOAA) decreased infarct size. As additional evidence, stroke patients with a poorer outcome had higher plasma cysteine levels (Wong
et al., 2006), and in the rat model the NMDA-receptor blocker MK-801 prevented the NaHS-induced damage (Qu et al., 2006).

A second proposed role of H$_2$S is in modulation of gamma-aminobutyric acid (GABA) receptors. Han et al. (Han et al., 2005) showed that in rats susceptible to febrile seizure exogenous addition of NaHS resulted in upregulation of GABA$_B$R1 and R2 (relative to the downregulation already present in the febrile seizure model), and treatment with hydroxylamine (HA), an inhibitor of PLP-dependent enzymes, downregulated GABA$_B$R2 but did not change expression of R1. Both types of receptors are required for a functional GABA channel, so a change in one will affect the overall GABA response. The ability of H$_2$S to increase GABA receptors will result in increased inhibitory transmissions. In a separate study (Han et al., 2005), the same authors showed that while giving NaHS or HA to rats did not affect the development of febrile seizures, NaHS did decrease histologic damage in the hippocampus while HA exacerbated the damage.

Peripherally, H$_2$S has been suggested to be a mediator of pain. Kawabata et al. (Kawabata et al., 2007) showed in rat paw model that injection of NaHS or cysteine caused hyperalgesia. The NaHS-induced pain was blocked by the oxidizing agent 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) and the T-type Ca$^{2+}$-channel inhibitor ethosuximide. The cysteine-induced hyperalgesia was blocked by propargylglycine (PPG) or β-cyanoalanine (BCA), both of which are CBS inhibitors. These two inhibitors also partially blocked the pain associated with lipopolysaccharide (LPS) injection. Using NG108-15 cells which only express T-type Ca$^{2+}$ channels, patch-clamping showed that
NaHS stimulated Ca\(^{2+}\) currents, which were abolished by DTNB. A model of pain associated with colitis, however, suggested that H\(_2\)S decreased nociception (Distrutti et al., 2006). This model used colon distension by a balloon to induce pain, which was decreased by NaHS or cysteine and enhanced by glibenclamide. The authors concluded that the pain modulation in this case occurred through K\(_{\text{ATP}}\) channels.

Kimura (Kimura & Kimura, 2004) found that NaHS administration prevented the cell death caused by glutamate-induced oxidative stress. This effect is due to the upregulation of glutathione (GSH) production via two mechanisms. The first is stimulation of \(\gamma\)-glutamylcysteine synthase, and the second is stimulation of the cystine/glutamate antiporter \(x_c^-\) to increase cystine in cells. The authors did not use any H\(_2\)S-synthesis inhibitors, and suggested that this effect could be physiologic based on their using H\(_2\)S concentrations in line with levels reported in the brain. *In vivo* it seems that because H\(_2\)S and GSH synthesis are both dependent on cysteine, situations in which GSH needed to be upregulated would also be situations in which cysteine was limiting.

Umemura and Kimura (Umemura and Kimura, 2007) found that 100 µM NaHS, but not GSH, dithiothreitol (DTT), \(\alpha\)-tocopherol, or 2-mercaptoethanol, increased the reducing capacity of primary rat neurons and murine Neuro2A. The effect was significantly stronger in these neuronal cells than other cell types, and was independent of inhibition of cytochrome-c and non-enzymatic reaction of the assay reagent, WST8, with NaHS. Also, the effect lasted up to 7 hrs after washout of the NaHS. The authors also showed that an increase in the NADH:total NAD ratio was not the reason for the increased reducing capacity, because 1mM NaN\(_3\) greatly increased the ratio while not
increasing the cells’ ability to reduce WST8. Yet another negative finding was that glibenclamide ($K_{\text{ATP}}$-channel inhibitor), $\text{LaCl}_3$ ($\text{La}^{3+}$ inhibits $\text{Ca}^{2+}$ channels), actinomycin D (mRNA synthesis inhibitor), and cycloheximide (protein synthesis inhibitor) did not alter the increased reducing capacity. The only treatment that decreased the response to NaHS was inhibition of tyrosine kinases, which led the authors to conclude that phosphorylation of tyrosines are involved in the activity and therefore NaHS might have a tropic effect in the brain.

Yet another proposed function of H$_2$S is in modulating Ca$^{2+}$ waves in glial cells (Nagai et al., 2004). In this case primary cultures of astrocytes were exposed to 20-160 $\mu$M NaHS after being stained with Oregon green (Ca$^{2+}$-sensitive fluorophore). Local application of NaHS caused Ca$^{2+}$ waves which propagated to neighboring cells. A similar response was seen in 400 $\mu$m sections of rat brain. Studies in K$^+$-free buffer and with thapsagargin (depletes intracellular Ca$^{2+}$ stores) showed that the Ca$^{2+}$ waves were dependent on both intra and extracellular Ca$^{2+}$, but more so extracellular. The specific type of Ca$^{2+}$ channel could not be determined, however. It was also found in this setup that NaHS did not activate $K_{\text{ATP}}$ channels. The authors showed that Ca$^{2+}$ release in isolated astrocytes could be stimulated by H$_2$S alone, and showed that in co-culture with neurons NMDA stimulated the neurons, which caused Ca$^{2+}$ waves in the astrocytes. The conclusion was that neuronal excitation caused Ca$^{2+}$ release via H$_2$S in astrocytes, but no evidence was presented directly showing an increase in H$_2$S with neuronal excitation.

H$_2$S has also been implicated in neural pathology. Eto et al. (Eto et al., 2002) found that in Alzheimer’s disease patients H$_2$S levels in the brain were decreased, and
suggested that H$_2$S could be related to some of the symptoms of Alzheimer’s. The authors did not propose a mechanism, however. Down’s syndrome (trisomy 21) has also been linked to endogenous H$_2$S, although in this case overproduction was blamed (Kamoun, 2001). The gene for CBS is found on chromosome 21 and is overexpressed in brains of Down’s patients (Ichinohe et al., 2005) and thus H$_2$S is in fact overproduced as well (Kamoun et al., 2003).

II.D.iv. Systemic Vasculature

The initial report implicating H$_2$S in vascular physiology was by Hosoki et al. in 1997 (Hosoki et al., 1997), who found that rat ileum, portal vein, and aorta all produced H$_2$S from cysteine and that H$_2$S could relax rat aorta alone or in synergy with nitric oxide (NO). Zhao et al. in 2001 (Zhao et al., 2001) followed this, and found that NaHS infusion decreased blood pressure in rats, and relaxed aortic rings in vitro, with an in vitro EC$_{50}$ of 125 µM. Using inhibitor studies, the authors eliminated prostaglandin, protein kinase C (PKC), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), superoxide, hydrogen peroxide, $K_{Ca}$ channels, and $K_V$ channels as potential targets, and determined that the vascular relaxation was via $K_{ATP}$ channel stimulation causing hyperpolarization. They then verified the role of $K_{ATP}$ channels using patch clamping studies. Plasma sulfide was measured to be ~45 µM using a sulfide ISE, thus implying a tonic action of H$_2$S on blood vessels. The action on $K_{ATP}$ channels has been confirmed by later studies (Tang et al., 2005).
The same lab next reported that the vascular relaxation was in part endothelium-dependent, and involved inhibition of charybdotoxin / apamin-sensitive $K_{Ca}$ channels in endothelial cells (Zhao & Wang, 2002). Cheng et al. (Cheng et al., 2004) reported a similar endothelial dependence on H$_2$S-induced relaxation in rat mesenteric arteries. The strongest evidence to date that H$_2$S is a physiological vasorelaxant has come from Yang et al. (Yang et al., 2008), who used CSE knockout mice to decrease endogenous H$_2$S levels. The authors found that CSE-deficient mice developed hypertension from an early age, with a concomitant decrease in plasma H$_2$S. In vitro, H$_2$S relaxed arteries that had been precontracted with phenylephrine more potently in CBS knockout than in wild type arteries ($IC_{50}$ of 75 µM vs. 120 µM), consistent with supersensitivity to sulfide due to decreased endogenous production. Conversely, methacholine-induced relaxation, which is endothelium-dependent, was diminished relative to wild-type. The endothelium-dependent portion of the H$_2$S relaxation was shown to be dependent on calcium-calmodulin stimulation of CSE (the same stimulus required for eNOS and heme oxygenase), which is dependent on Ca$^{2+}$ release through inositol 1,4,5-triphosphate (IP$_3$). Interestingly, it has been recently shown in human saphenous vein primary endothelial cells that the reciprocal may also be true, i.e. that H$_2$S ($\geq$50 µM) can stimulate IP$_3$-dependent release of Ca$^{2+}$ from intracellular stores (Bauer et al., 2009). This then presents the possibility of a positive feedback loop.

Some manner of interaction certainly exists between H$_2$S, NO, smooth muscle cells, and endothelium, although the research is not clear cut on the matter. Zhong et al. (Zhong et al., 2003) reported that exogenous NaHS could partially reverse the
hypertension caused by chronic nitro-L-arginine-methyl-ester (L-NAME)-induced inhibition of nitric oxide synthesis and decrease ventricular hypertrophy, and that the decreased NO (as opposed to the L-NAME itself) inhibited CSE expression, thus inhibiting H₂S synthesis. This led to the conclusion that NO may stimulate H₂S synthesis and that dysfunction of the H₂S-synthesizing pathways could contribute to hypertension, consistent with the conclusion of Yang et al. (Yang et al., 2008). It has also been reported that H₂S in “low” doses (100-300 µM in rat aorta, 30-100 µM in mouse aorta) can cause vasoconstriction by directly inhibiting eNOS (Kubo et al., 2007), an effect which was eliminated by removing the endothelium. A similar but longer-term effect was demonstrated by Geng et al. (Geng et al., 2007), who showed that H₂S downregulated the L-arginine/eNOS/NO system in rat aorta and human umbilical vein endothelial cells (the HUVEC cell line). H₂S has also been found, however, to indirectly stimulate NO and iNOS by potentiating IL-1β-induced NF-κβ activation while having no direct effect on NO. A direct interaction of NO with H₂S has been proposed, though, with the formation of a nitrosothiol eliminating the effect of both gasses (Ali et al., 2006). The converse has also been shown, however, with H₂S causing the release of NO from various nitrosothiols (Ondrias et al., 2008).

Although much less discussed, H₂S may also cause vasoconstriction. Koenitzer et al. (Koenitzer et al., 2007) showed that at higher oxygen tensions (200 µM) H₂S caused vasoconstriction in partially precontracted rat aorta, and suggested (although did not support with any data) that this response was related to an oxidation product and not H₂S itself. It has also been shown that H₂S (5-100 µM) can reverse the β-blocker-
induced relaxation of phenylephrine-precontracted rat aorta, and the authors show that this effect is related to decreasing cAMP accumulation (Lim et al., 2008). Similarly, Webb et al. (Webb et al., 2008) have shown that H₂S reverses the acetylcholine-induced relaxation in phenylephrine-contracted human internal mammary arteries, but in this case relate the effect to interference with NO or eNOS. Generally it appears that H₂S does not contract vessels that have not been prestimulated, with a notable exception being cyclostome vessels, as reported by our lab (Olson et al., 2001).

Aside from the above descriptions, H₂S has been implicated in indirect vasodilation via direct inhibition of angiotensin converting enzyme (Laggner et al., 2007), as a vasodilatory signal from perivascular adipose tissue (Fang et al., 2009), and as proangiogenic via Akt phosphorylation (Cai et al., 2007) and K<sub>ATP</sub> / MAPK stimulation (Papapetropoulos et al., 2009). One study has suggested that H₂S may relax vascular smooth muscle by stimulating the Cl⁻ /HCO₃⁻ exchanger and thus causing intracellular acidification (Lee et al., 2007). Finally, H₂S-induced vasodilation has been credited with some of the cardiovascular benefits of garlic (Benavides et al., 2007).

II.D.v. Heart

One of the most active areas of hydrogen sulfide research has been on its beneficial effects on cardiac function in the face of ischemia and/or reperfusion injury. The first report of this relationship was by Geng et al. in 2004, who showed that in sub-chronic isoproterenol-induced ischemic conditions, plasma H₂S decreased while CSE expression was upregulated, and that giving exogenous NaHS decreased mortality and
signs of cardiac inflammation (Geng et al., 2004). This effect was proposed to be related to reactive oxygen species (ROS) scavenging, and other studies have found an antioxidant effect of H₂S in the heart (Su et al., 2009). Bian et al. found that preconditioning with NaHS increased function and viability in an ischemia/reperfusion model, while ischemic preconditioning in the face of PPG administration had the opposite effect (Bian et al., 2006). The authors attributed the action of NaHS to PKC and sarcolemmal KᵦTP channel activation. The relevance of sarcolemmal KᵦTP channels to the beneficial actions of NaHS was also described by Pan et al. in a primary cardiomyocyte model of myocardial infarction (MI), in which it was likewise found that PPG could decrease the benefit of preconditioning (Pan et al., 2006).

Also in 2006, it was shown that 5-hydroxydecanoate, a blocker of mitochondrial KᵦTP channels, could prevent the beneficial effect of NaHS given prior to left anterior descending artery (LAD) occlusion in in vivo rat hearts, and that PPG increased infarct size relative to control, implying that endogenous H₂S had a protective role in ischemia via mitochondrial KᵦTP channels (Sivarajah et al., 2006). This finding was confirmed in an ex vivo rat heart model of ischemia/reperfusion in which the hearts were perfused 10 minutes before, then during LAD occlusion, and 10 minutes following initiation of reperfusion (Johansen et al., 2006). Sivarajah et al. have since reported similar findings, adding that NaHS preconditioning also reduced many markers of inflammation and apoptosis (Sivarajah et al., 2009). It is also possible that cell membrane KᵦTP channels could be involved in H₂S-related cardioprotection, as shown by patch clamping studies (Zhong et al., 2009).
Many other groups have reported the benefits of NaHS (both endogenous and exogenous) preconditioning in ischemia/reperfusion injury, and have additionally attributed them to ERK1/2 and PI3K (Hu et al., 2008), PKC phosphorylation (Calvert et al., 2009; Pan et al., 2009), and Nrf2 nuclear localization (Calvert et al., 2009). S-allylcysteine, a compound found in garlic, decreases infarct size while increasing plasma H$_2$S, and PPG blocks these effects, leading the authors to conclude that some of the cardioprotective effects of garlic are related to H$_2$S formation in the heart (Chuah et al., 2007). Identical results were found with S-propargyl-cysteine, a structural analog to S-allylcysteine (Wang et al., 2009). In contrast to all of the above, one study, while agreeing that PPG increased infarct size, found that exogenous NaHS did not decrease infarct size and endogenous H$_2$S synthesis was not involved in cardioprotection (Bliksoen et al., 2008).

Sulfide given after the onset of ischemia or early during reperfusion has been shown to decrease injury after ischemia as well, and is more clinically relevant. Elrod et al. were the first to report this, showing that H$_2$S (as an unspecified “H$_2$S donor”) given at the onset of reperfusion decreased infarct size and increased left ventricular function, which the authors attributed to preservation of mitochondrial function (Elrod et al., 2007). The same effect was shown by Zhang et al., although in this case the effect was attributed to $K_{\text{ATP}}$ channel activation (Zhang et al., 2007). Later work has shown that ischemic postconditioning upregulates CSE and can be inhibited by PPG, leading to the conclusion that it involves endogenous H$_2$S, and that the benefit of endogenous or exogenous H$_2$S is related to activation of Akt and PKC-α and PKC-ε (Yong et al., 2008).
One of the benefits of H$_2$S treatment with respect to reperfusion injury is a decrease in cardiomyocyte apoptosis (Sodha et al., 2008), which is likely related to inhibition of c-Jun N-terminal kinase (Shi et al., 2009) and upregulation of the survivin gene (Zhuo et al., 2009). In one of the most practical demonstrations of hydrogen sulfide’s benefits, mice were given KCl to induce asystole for seven minutes, after which they were given an infusion of NaHS for 1 min and then during CPR until spontaneous return of circulation (Minamishima et al., 2009). NaHS-treated mice had a much higher survival at 24 hr, and mice overexpressing CSE had better outcomes as well. In these mice NaHS stimulated nitric oxide synthase 3 (NOS3), and genetic deficiency of NOS3 eliminated the beneficial effect of NaHS, leading the authors to conclude that the benefit of NaHS was mediated through NOS3. In contrast to the above, a more recent study in a porcine model of cardiac ischemia/resuscitation found that administration of Na$_2$S during resuscitation actually resulted in worse outcomes than control (Derwall et al., 2010).

Other aspects of hydrogen sulfide-related cardiac physiology are not well studied, with only two studies of which I am aware addressing the topic. Endogenous H$_2$S seems to have a negative inotropic effect (Geng et al., 2004), and it is able to inhibit L-type calcium channels in cardiomyocytes (Sun et al., 2008).

II.D.vi. Immunology / Shock

Hydrogen sulfide appears to have a role in inflammation and shock, although what that role might be is not clear. In many studies involving a cecal ligation and puncture (CLP) model of sepsis, H$_2$S appears to be pathologic. Hui et al. found that
tissue H₂S increases in CLP as well as endotoxin-induced sepsis (Hui et al., 2003), and
Zhang et al., in a series of studies, found that H₂S increased and PPG decreased lung
production of substance P (Zhang et al., 2007), NF-κβ and various cytokines (Zhang et
al., 2007) via ERK phosphorylation (Zhi et al., 2007; Zhang et al., 2008) and leukocyte
rolling and adhesion molecules (Zhang et al., 2007). Also in a CLP model, Ang et al.
proposed that H₂S-induced sepsis exacerbation was mediated through transient receptor
potential vanilloid 1 (TRPV1) receptors (Ang et al.).

H₂S also seems to be considered pathologic in an endotoxin/LPS model of sepsis.
Its plasma levels are increased in this model as well as in humans with septic shock, and
intraperitoneal H₂S injection exacerbated shock-related lung damage (Li et al., 2005a).
Addition of PPG has been shown to decrease organ damage from LPS (Collin &
Thiemermann, 2005). The glucocorticoid dexamethasone decreases LPS-induced
inflammation, and also decreases the LPS-induced rise in H₂S (Li et al., 2008). On the
other hand, Hu et al. showed in microglial cells that H₂S decreased LPS-induced NO
release, as did S-adenosylmethionine, a stimulator of CBS (Hu et al., 2007). PPG and
AOAA had the opposite effect, and the authors attributed this in part to the p38 / MAPK
pathway.

In more localized models of inflammation, H₂S tends to act more as an anti-
inflammatory. Carrageenan-induced paw edema was decreased by injection of NaHS and
enhanced by PPG, and NaHS also decreased aspirin-induced leukocyte adhesion to
endothelium (Zanardo et al., 2006). In the same inflammation model, S-diclofenac, a
nonsteroidal anti-inflammatory drug which releases H₂S in vivo, decreased inflammation
relative to control and relative to the anti-inflammatory parent molecule, diclofenac (Sidhapuriwala et al., 2007). In other experiments by the same group, cerulein-induced pancreatic (and resulting lung) inflammation, chemokine production, and adhesion molecules are all decreased by intraperitoneal (IP) injection of NaHS (Sidhapuriwala et al., 2009).

Hemorrhagic shock may also be influenced by H$_2$S. In one study it was shown that plasma H$_2$S increased after hemorrhage in a rat model, and the treatment with PPG partially restored mean arterial pressure and heart rate (Mok et al., 2004). There were no data on survival presented, however. The same authors later replicated their earlier results and showed that when given PPG prior to hemorrhage, rats recovered more quickly and showed fewer signs of inflammation than controls after return of the withdrawn blood (Mok & Moore, 2008). The authors suggested that inhibition of H$_2$S synthesis may be a treatment for hemorrhagic shock, but again did not provide mortality data. Perhaps counterintuitively based on the preceding studies, it has been suggested that giving H$_2$S may actually be a treatment for hemorrhagic shock. Morrison et al. have reported that when rats were treated with inhaled or IV H$_2$S after 60% blood loss, survival increased dramatically from ~20% to ~70% (Morrison et al., 2008). The rational for such dramatic results involved the overall decrease in metabolism and ‘suspended animation’ state induced by high levels of H$_2$S as described by Blackstone and Roth (Blackstone & Roth, 2007).
II.D.vii. Genitourinary

In the kidney, hydrogen sulfide has been shown to increase glomerular filtration rate (GFR) and sodium and potassium excretion, while blockage of renal H$_2$S production by PPG + AOAA decreases them (Xia et al., 2009). The increase in GFR was attributed to vasodilation, while the tubular effects were attributed to inhibition of the tubular Na$^+$/K$^+$/2Cl$^-$ cotransporter and Na$^+$/K$^+$ ATPase. H$_2$S has also been shown numerous times to decrease renal ischemia/reperfusion injury. Tripatara et al. found a decrease in injury markers after reperfusion in the presence of NaHS, and an increase when endogenous production was blocked by PPG (Tripatara et al., 2008). This was confirmed in a similar report, in which the protective effect of exogenous H$_2$S was attributed to its ability to lower metabolic demand (Bos et al., 2009). Xu et al. found that ischemia/reperfusion actually decreased CBS activity, leading to reperfusion injury, which could be ameliorated with injection of a nitric oxide scavenger (which increased H$_2$S levels) or NaHS (Xu et al., 2009b). In a porcine non-beating heart donor model, kidneys were subjected to 15 minutes warm ischemia followed by 18 hr cold storage, after which they were perfused with 0.5 mM NaHS for 10 minutes before and after reperfusion with autologous blood (Hosgood & Nicholson, 2009). The sulfide-exposed kidneys had higher glomerular filtration rate and creatinine clearance and less oxidative injury than controls.

Bladder may also be affected by H$_2$S. TRPV1 receptors are present on uroepithelium and bladder afferent nerves, which can respond to H$_2$S to cause an increase in micturition frequency (Streng et al., 2008). This has been shown in other work as
well, although whether the responsive receptor was TRPV1 or another TRP receptor was questioned (Patacchini et al., 2004; Patacchini et al., 2005). The prostate may contain a sulfide-sensitive TRP receptor, as well (Gratzke et al., 2009). Work in our lab has found in trout bladder, contrary to the contractile responses above, that H$_2$S is a relaxant, and may function oxygen-dependently (Dombkowski et al., 2004). Relaxation in rat vas deferens has also been attributed to H$_2$S (Teague et al., 2002).

In a paper that garnered some popular press, d’Emmanuele di Villa Bianca et al. demonstrated that H$_2$S was produced in and could relax human corpora cavernosa tissue and could cause penile erections in rats (d'Emmanuele di Villa Bianca et al., 2009). Similar findings had previously been reported for male (Srilatha et al., 2007) and female (Srilatha et al., 2009) erectile tissue in rabbits.

II.D.viii. Gastrointestinal

The colon is unique in the body with respect to hydrogen sulfide, because it is exposed to both endogenously produced H$_2$S as well as much higher levels that are produced by gut microbes. Normally the colonic mucosa oxidizes H$_2$S very quickly, a function that has been attributed primarily to the enzyme rhodanese (Furne et al., 2001; Picton et al., 2002). This is important because higher levels of H$_2$S can prevent colonic epithelial apoptosis, which may increase cancer risk (Rose et al., 2005), and H$_2$S has also been associated with ulcerative colitis (Rowan et al., 2009). In fact, a recent colitis model found that rhodanese is upregulated in colitis, and that inadequate upregulation
may exacerbate the colitis by allowing excess accumulation of $H_2S$ (Taniguchi et al., 2007; Taniguchi et al., 2009).

Endogenous hydrogen sulfide has been implicated in normal gut physiology as well. Rat, mouse, guinea pig, and human colon have all been shown to produce $H_2S$ (Martin et al., 2009; Schicho et al., 2006). Its suggested functions have included direct modulation of smooth muscle contractility (Teague et al., 2002), modulation of secretory activity via activation of TRPV1 receptors (Schicho et al., 2006), and both anti-nociception through action of $K_{ATP}$ channels (Distrutti et al., 2006) and pro-nociception in the dorsal root ganglion (Xu et al., 2009a) and via T-type calcium channels in primary afferent neurons (Matsunami et al., 2009).

In the stomach, $H_2S$ has been suggested to play roles in both increasing and decreasing mucosal damage. Medieros et al. showed that NaHS and cysteine decreased gastric damage caused by intragastric injection of 50% ethanol, an effect which was prevented by glibenclamide, PPG (with cysteine), or capsazepine, which blocks sensory afferent neurons containing TRPV1 receptors (Medeiros et al., 2009). The authors concluded that $H_2S$ may decrease gastric injury both directly by acting on $K_{ATP}$ channels and indirectly by stimulating TRPV1 receptors. Subsequently, a different group reported the opposite effect when absolute ethanol was used, that NaHS was not protective and that administration of PPG actually decreased gastric damage (Chavez-Pina et al., 2009). The authors suggest that the difference between the two studies may be attributed to the different concentration of ethanol used. In an acetic acid model of gastric ulcers, daily administration of NaHS sped up the healing process, an effect which was mimicked by
cysteine and abolished by PPG (Wallace et al., 2007). In this case the authors found no relationship to $K_{\text{ATP}}$ channels or NOS. H$_2$S has also been shown to decrease gastric damage caused by nonsteroidal antiinflammatories (NSAID), which themselves may exacerbate that effect by inhibiting production of H$_2$S at the Sp1 promoter site of CSE (Fiorucci et al., 2005).

II.D. ix. Pulmonary

Hydrogen sulfide has been proposed as a factor in decreasing the pulmonary remodeling that accompanies pulmonary hypertension, either that caused by hypoxia (Jin et al., 2006) or surgically-created high blood flow (Li et al., 2005). Production of H$_2$S appears to be downregulated in pulmonary hypertension (Zhang et al., 2003), and intraperitoneal NaHS administration can help prevent some of the associated chronic changes (Li et al., 2006). Zhang et al. have also shown that giving NaHS in the setting of chronic hypoxic pulmonary hypertension decreased pathologic remodeling, while administering PPG worsened it (Zhang et al., 2003; Zhang et al., 2004). Another study has shown that giving L-arginine in high blood flow pulmonary hypertension upregulates CSE/H$_2$S, decreasing remodeling (Wang et al., 2006). Similarly, in a murine bleomycin model of pulmonary fibrosis, intraperitoneal injection of NaHS has been shown to decrease fibrosis and migration of pulmonary fibroblasts, with a mechanism involving ERK phosphorylation but unrelated to $K_{\text{ATP}}$ channels (Fang et al., 2009; Fang et al., 2009). Our lab has proposed that an increase in H$_2$S during acute hypoxia causes pulmonary vessels to constrict, which is contrary to the above reports (Olson et al.,
The discrepancy is likely due the timeframe, ours looking at acute changes and others looking at chronic changes.

A decrease in pulmonary H$_2$S has been associated with an ovalbumin model of asthma, the inflammatory changes of which could be reduced by exogenous NaHS (Chen et al., 2009a). Inflammation induced by burn/smoke inhalation in sheep and mice may also be decreased by administration of NaHS, with a resulting decrease in mortality (Esechie et al., 2008; Esechie et al., 2009). Perhaps through related mechanisms, NaHS infusion has been shown to decrease ischemia/reperfusion injury in lungs (Fu et al., 2008), a finding that may also be related to the ability of hydrogen sulfide to act as an antioxidant in the lungs (Wei et al., 2008).

Decreased plasma H$_2$S has been proposed as a marker of community acquired pneumonia (Chen et al., 2009) and COPD (Chen et al., 2005), although with the >10-fold range of reported ‘normal’ plasma levels (Appendix I) it is difficult to imagine this coming into widespread use.

Finally, minimal work has been done investigating the relevance of H$_2$S to airway smooth muscle function, although one study has reported a physiologic bronchodilator function (Kubo et al., 2007).

II.D. c. Endocrine

The pancreatic effects of hydrogen sulfide generally appear to involve apoptosis and inhibition of insulin secretion. Cao et al. (Cao et al., 2006) demonstrated that incubation in 10 µM NaHS for 3 hr resulted in acinar cell apoptosis (acinar cells are not
endocrine but are included here). Yang et al. found that H₂S could induce apoptosis in an insulin-secreting β-cell tumor line, as could overexpression of CSE (Yang et al., 2007). The effect was related to endoplasmic reticulum stress, involving the p38 / MAPK pathway. Conversely, Kaneko et al. found that apoptosis induced in freshly isolated β-cells by high glucose was inhibited by NaHS or cysteine, and that under conditions of high glucose CSE was upregulated (Kaneko et al., 2009). The authors’ explanation for this effect was that H₂S may scavenge ROS resulting from the excess glucose. The discrepancy between their work and that of Yang et al. was proposed to be related to the former’s use of a tumor cell line.

H₂S consistently decreases insulin secretion. Ali et al. showed that the decrease in insulin production from a β-cell tumor line could be inhibited by glibenclamide, and saw no change in reduced glutathione or ATP levels (Ali et al., 2007). Mouse islet cells responded the same way to NaHS as well as to cysteine. This study, however, did see a decrease in intracellular ATP. Evidence for the physiological role of H₂S in insulin regulation comes from a study of Zucker diabetic fatty rats, which have increased plasma H₂S and decreased insulin, a relationship that is reversed after 4 weeks of intraperitoneal injections of PPG (Wu et al., 2009). The same study found in isolated β-cells that H₂S increased potassium currents, suggesting an effect of K⁺ATP channels, which are known to be involved in insulin secretion (Remedi & Koster, 2009). Increased plasma H₂S has also been found in rats with diabetes induced by streptozocin administration (Yusuf et al., 2005).
Data on other endocrine functions of H$_2$S are very limited. Dello Russo et al. (Dello Russo et al., 2000) propose that stress-induced, but not basal, corticotropin releasing hormone release is down-regulated by increasing H$_2$S levels. The authors tested both NaHS and S-adenosyl methionine in hippocampal slices from rats. NaHS had to be at a level of at least 1 mM to have this effect, however, and thus it is difficult to imagine that this is a physiologic situation. Eto and Kimura (Eto & Kimura, 2002) have shown that testosterone can increase SAM in brain, which increases H$_2$S. In the only non-mammalian description of which I am aware, Perry et al. found that H$_2$S could induce catecholamine secretion from chromaffin cells of trout (Perry et al., 2009).

III. Thesis rational

When I joined our lab, previous work there, based on vessel myography, had shown that H$_2$S and hypoxia had very similar effects on vessel tension and that during stimulation by one, the action of the other was generally prevented or greatly attenuated when applied subsequently. Crucially, this held true in both vessels which contracted and vessels which relaxed to hypoxia. Also, addition of inhibitors of H$_2$S synthesis eliminated or greatly diminished vessels’ normal response to hypoxia while not significantly altering their response to exogenously applied H$_2$S. These results suggested that H$_2$S and hypoxia were acting through the same pathway. Additional published research had shown the presence of H$_2$S in blood and tissues and that H$_2$S production could be measured in tissues, as well as having shown the ability of mitochondria to consume H$_2$S oxygen-dependently. This led Dr. Olson and his student at the time, Ryan
Dombkowski, to hypothesize that H$_2$S could act as an oxygen sensor: during normoxia consumption of H$_2$S balanced production and thus no H$_2$S accumulated, while during hypoxia H$_2$S consumption was inhibited and thus H$_2$S could accumulate and exert its effect. While this work was convincing, there was still a lack of direct experimental evidence for the inverse relationship between O$_2$ and H$_2$S. Experiments to address this would require techniques beyond vessel myography. Additionally, no attempts have been made to compare consumption rates to production rates with respect to oxygen, which should enable conclusions to be made about the likelihood of H$_2$S acting physiologically.

III.A. H$_2$S Sensor Development

The ideal system for investigating the relationship between O$_2$ and H$_2$S would be a combination of a standard Clark-type oxygen sensor and a real-time H$_2$S-specific sensor which could be used in physiologic conditions. A recent paper had described the design of just such an amperometric H$_2$S sensor and indicated its commercial availability. Our lab purchased one, which I used in early experiments with a fair amount of difficulty. In the process of reverse-engineering the sensor to determine how to connect it to the amperometric amplifiers available to our lab, it became clear that its design was in fact very simple and could be amenable to in-house construction. Considering the unimpressive performance and the cost of the commercial sensor (~$700) as well as the cost and fragility of the replacement membranes (~$40 each), I determined that it would be worthwhile to construct the sensor myself.
III.B. Inconsistencies in blood measurements

Blood would be the simplest place to look for changes in H$_2$S levels with varying oxygen, and certainly would be the simplest for *in vivo* studies. For the past 10 years or so it has been considered fact that the circulating [H$_2$S] is at least 10 µM, and generally higher. Since the year 2000 a minimum of 40 reports have corroborated this, with a range of 10 to ~300 µM reported for plasma sulfide (Appendix I). This, and similar levels in tissue, have generally been the justification for calling experimental levels of H$_2$S “physiologic”. Preliminary experiments in trout blood with the H$_2$S sensor, however, indicated that plasma H$_2$S was actually far lower than these recent reports state. An in-depth literature review found that papers published prior to 2000 almost invariably supported my preliminary findings. There is also a substantial body of literature spanning the past century that describes the rapid catabolism of sulfide in the blood and organs (see above and Discussion). This convinced me that a more detailed study of H$_2$S in the blood was in order. The H$_2$S sensor adds an important piece to the puzzle because all prior studies relied on some sort of chemical manipulation of the sample and were based on time-point measurements. My study also included the two techniques for detection of sulfide most commonly used in contemporary physiological experiments, the methylene blue method and the ion-selective electrode.
III.C. Inverse Relationship Between Oxygen and Hydrogen Sulfide.

Despite the vast number of papers describing the myriad physiologic functions of H\textsubscript{2}S, almost none of them mention the somewhat obvious potential for H\textsubscript{2}S to act oxygen-dependently. The fact that H\textsubscript{2}S is oxidized to sulfate, using O\textsubscript{2}, has been known at least since 1921 when the oxidation of H\textsubscript{2}S in blood was described by Haggard (Haggard, 1921). Smythe (Smythe, 1942) also noted that two products of cysteine desulfuration by liver, ammonia and pyruvate, were formed in equal amounts aerobically or anaerobically (which supports our hypothesis of constitutive production), while the recovered H\textsubscript{2}S was much lower in aerobic experiments. It seems as though this relationship was taken as common knowledge prior to the description of H\textsubscript{2}S as physiologically relevant, but was ignored afterward. To my knowledge our lab was the first to link the two (Olson \textit{et al.}, 2006), with only two subsequent papers directly addressing the issue, one of which our lab coauthored (Koenitzer \textit{et al.}, 2007; Olson \textit{et al.}, 2008). The production and consumption rates of H\textsubscript{2}S had not been compared, however, so there was no way to establish the physiological relevance of the relationship. The absence of measurable sulfide in the blood required the use of tissues to answer this question. My approach was to establish conditions that favored production of hydrogen sulfide by tissue homogenates and investigate how changing the ambient oxygen changed the hydrogen sulfide’s accumulation or consumption, as measured with the amperometric sensor.
III.D. PEBBLEs

The literature on hydrogen sulfide contains many reports describing the production of H$_2$S by tissues, but to my knowledge none, including those from our lab and data presented in this thesis, use physiologic conditions. One concern is that tissues usually are homogenized during or after the experiment. A second concern is the supraphysiologic concentration of cysteine needed to demonstrate production (typically $\geq$1 mM). In researching potential solutions to this problem, it became clear that an ideal means for measuring this would be to use an intracellular marker of H$_2$S that could be microscopically visualized in living cells. Photonic Explorers for Bioanalysis with Biologically Localized Embedding (PEBBLE) looked to be the most promising means of accomplishing this, and have been used to detect various ions as well as CO$_2$ and O$_2$ (Koo et al., 2004). PEBBLEs are nanoscale (hundreds of nanometers in diameter) beads that contain a stable reference fluorophore and an analyte-sensitive detection fluorophore. They are produced from either organically modified silicates (ormosil) or acrylamide, the monomers of which are polymerized in the presence of the two dyes to form the porous, dye-containing beads. Once inserted into cells through one of a few different techniques, changes in the ratio of fluorescence intensities of the two dyes are measured and correspond to changes in analyte concentration. A sulfide-sensitive dye (fluorescein mercuric acetate) already existed and the production of PEBBLEs is relatively straightforward, thus I attempted to develop a PEBBLE-based probe for intracellular H$_2$S detection.
MATERIALS AND METHODS

I. Animals, blood, and tissue sampling

All animal protocols were approved by institutional IACUC review. All blood was collected in heparinized (~50 USP/ml) syringes or plastic containers. Tissues were kept on ice in the appropriate buffer.

Sea lamprey (*Petromyzon marinus*, 0.13-0.45 kg) were trapped in streams feeding into the Great Lakes and maintained at Indiana University School of Medicine - South Bend (IUSM-SB) in 500 l tanks with aerated, through-flowing well water (14°C) and a 12:12-hr light:dark cycle. For blood collection they were anesthetized in benzocaine (1:5000 wt/vol), opened with a ventral mid-line incision, and blood was drawn from the posterior cardinal veins with a curved 21G needle, bevel up. Hearts were excised after anesthesia with a ventral mid-line incision of the thoracic cavity. Dorsal aortae were excised after anesthesia by removing the abdominal wall by cutting perpendicularly across the abdomen near the heart and then cutting down the length of the abdominal cavity on both sides. The resulting skin flap was pushed aside and the abdominal viscera removed. A lengthwise incision was then made along each cardinal vein just lateral to either side of the dorsal aorta. The dorsal aorta could then be cut proximally and pulled free down the length of the animal.
Rainbow trout (*Oncorhynchus mykiss*, 0.4-0.7 kg) were obtained from a commercial hatchery and maintained in circulating 2000 l tanks under the same conditions as lamprey. Blood was drawn from the hemal arch of lightly restrained fish by inserting a 20G needle ventrally along the midline, just caudal to the anal fin. Fish were held through paper towels to minimize contact with their skin, and returned to the tank after the draw. Ventricles were obtained after killing the fish with a blow to the head and opening the thoracic cavity along the midline. Steelhead ventricles were obtained from fish at Bodine State Fish Hatchery anesthetized in MS-232 for the purpose of spawning. Trout gills were obtained from fish killed by a blow to the head. The thoracic cavity was opened and the ventricle injected with 1 ml of heparinized saline. The ventricle was then removed and the ventral aorta cannulated with polyethylene tubing, which was sutured in place. The gills were perfused through the ventral aortic cannula with cold heparinized saline until no more blanching was seen in the gill filaments (typically a few hundred ml). The first arches were then removed.

Lobund-Wistar (LW) rats (0.35-0.5 kg), Harlan-Sprague-Dawley (HSD) rats (0.4-0.5 kg), and C57 Black/6 mice (~25 g) were housed on-site and kept on a 12:12 hr light:dark cycle with access to food and water *ad libitum*. LW rats and mice were euthanized with CO₂ and blood drawn via cardiac puncture while the heart was still beating. HSD rats were heparinized I.P. with 0.1 ml of 10 mg ml⁻¹ heparin and then anesthetized with 1 ml of 50 mg ml⁻¹ pentobarbital. Blood was drawn from the hepatic vein (flow cell experiments) or thoracic cavity (metabolism experiments).
Pig (*Sus scrofa*) and cow (*Bos taurus*) mixed venous and arterial blood was collected in heparinized containers within 5-10 min after the animals were killed at a local slaughterhouse. Blood was used within 1 min after collection for flow cell experiments and within 8 min for diffusion chamber experiments; blood for H$_2$S consumption studies was kept on ice and used the same day. Heparin did not interfere with H$_2$S measurements or H$_2$S consumption. Bovine heart and lung tissue was obtained immediately after removal of the organs from the animal at the slaughter house, and coarsely minced on ice in Kreb’s buffer to speed cooling. Tissue was transported back to the lab (~45 minute trip) on ice.

II. Cell culture

Bovine pulmonary artery smooth muscle cells (PAMSC) were donated by Dr. Jane Madden of the Medical College of Wisconsin. Cells were maintained in Cascade Biologics Smooth Muscle Cell Medium 213 with SMC supplement, in an incubator at 37°C with an atmosphere of 5% CO$_2$. No antibiotics were used for routine culture. Cells that were used in PEBBLE experiments were trypsinized, washed, and added to 35 mm culture dishes with a plain glass coverslip on the bottom. In experiments in which cells were exposed to non-sterile conditions and not fixed immediately the medium contained pen/strep and amphotericin. Cells that were used in the sensor chamber were trypsinized and resuspended in a volume of medium sufficient for the relevant experiment. Human MC07 breast cancer cells were donated by Dr. Tracy Vargo’s lab and plated on 12 mm poly-L-lysine-coated coverslips in Dubelco’s Modified Eagle’s Medium (DMEM) with
10% fetal bovine serum (FBS). These cells were used in PEBBLE experiments because they exhibited much less autofluorescence than our stock of PASMC.

III. Isolation of red blood cell ghosts.

Red blood cell ghosts were isolated from cow blood as described previously (Marchesi & Palade, 1967). Following isolation, an aliquot of ghosts was washed in Kreb’s buffer and another in potassium phosphate buffer, both pH 7.4, and resuspended 1:1 in the same buffer as the wash.

IV. Amperometric H$_2$S Measurements.

An amperometric H$_2$S sensor (AHSS) was constructed after Doeller et al. (Doeller et al., 2005), with the following modifications (Whitfield et al., 2008). A 32 G platinum wire anode was melted at the tip to form a bead, heat-sealed in a borosilicate capillary tube, and the tip ground to the widest point of the bead (~500 µm) and polished. In later versions (Figure 2) the anode consisted of a 26 G Pt wire heat-sealed over the distal ~5 mm of a 15 mm length of soda lime glass capillary tube, with 0.02 in I.D. x 0.037 in O.D., with silastic tubing inserted over the proximal end of the capillary tube and insulating the anode. This method proved to be both simpler to construct and less breakable. The remainder of the construction was similar for all versions. A 32 G platinum wire cathode was coiled around the capillary tube (10-15 wraps), and the whole assembly inserted into an outer glass tube 2 mm in diameter, containing a filling solution of 50 mM potassium ferricyanide in pH 10, 0.5 M sodium carbonate buffer. An O-ring
Figure 2. Schematic of final amperometric hydrogen sulfide sensor (AHSS) design, not precisely to scale. The anode (a) consisted of a 26G platinum wire heat-sealed over the distal ~5 mm of a 15 mm length of soda lime glass capillary tube (gct), with 0.02 in I.D. x 0.037 in O.D. Silastic tubing (St) inserted over the proximal end of the capillary tube and insulating the anode. A 32 G platinum wire cathode (c) was coiled around the capillary tube (10-15 wraps), and the whole assembly inserted into an outer glass case (ogc) 2 mm in diameter, containing a filling solution of 50 mM potassium ferricyanide in pH 10, 0.5 M sodium carbonate buffer. An O-ring seated in a groove (g) in the outer glass case to hold in place a 25µm-thick silicone polycarbonate membrane (SSP-M213; Specialty Silicone Products, Inc, Ballston Spa NY).
seated in a groove in the outer glass case to hold in place a 25µm-thick silicone polycarbonate membrane (SSP-M213; Specialty Silicone Products, Inc, Ballston Spa, NY) and in conjunction with a threaded sleeve secured it in the flow-through cell or metabolism chamber (see below). The sensor was connected to an Apollo 4000 Free Radical Analyzer (WPI, Sarasota FL) with 100 mV polarizing voltage.

As the amperometric H₂S sensor measures only H₂S gas, it was necessary to measure the pH of each sample and calculate the total sulfide from the Henderson-Hasselbalch equation using a pKₐ of 6.6 (37°C, 140 mM NaCl; mammalian blood) and 6.9 (15°C, 140 mM NaCl; fish blood) after Hershey et al. (Hershey et al., 1988). It should be noted that the pKₐ of 6.6 at 37°C results in 14% of total sulfide being present as H₂S, half of the more commonly cited value of 30% (Lowicka & Beltowski, 2007; Qu et al., 2008) which was derived from the pKₐ of H₂S at 20°C in water (7.05; (Lide, 1998). The detection limit of our polarographic H₂S sensor was 14 nM H₂S gas, i.e. 100 nM total sulfide at pH 7.4, 37°C, and 115 mM NaCl. H₂S gas consumption by the sensor at 37°C in 1.0 ml of Kreb’s buffer spiked to10 µM Na₂S was 3 pmol min⁻¹; less than a 0.1% min⁻¹ decrease in concentration.

V. Sulfidostat

In order to determine oxygen and H₂S consumption rates at (approximately) steady-state H₂S levels, a system was constructed similarly to the ‘sulfidostat’ described by Searcy and Peterson (Searcy & Peterson, 2004). The analog H₂S sensor BNC output from the Apollo 4000 was interfaced to a laptop PC USB port through a Measurement
Computing Personal Measurement Device A-D converter. A program was written in SoftWire which read the AHSS output at 1 Hz and compared it to a user-controlled set point. When the output fell below the setpoint, the program instructed a Harvard Apparatus syringe pump to meter out a designated volume Na$_2$S solution. This setup maintained the H$_2$S concentration to within approximately ±0.25 µM. Figure 3 shows a 10 µM Na$_2$S spike added in a single dose by syringe followed by a wash and then a series of five 1 µM and one 5 µM injections from the sulfidostat.

VI. Flow cell.

An acrylic water-jacketed flow cell (chamber volume ~5 µl, Figure 4) was constructed for rapid polarographic measurement of H$_2$S in blood. Blood was drawn directly from the sampling syringe through the cell at 0.5 ml min$^{-1}$ with a syringe withdrawal pump within one minute of removal from the animal. A second sample from the same animal (a second animal if mouse) was spiked with Na$_2$S (10 µM total sulfide concentration) and passed through the flow cell to verify that the system was responsive. Iodometric titration of a 100 µM standard in Kreb’s buffer, pH 7.4, before and after passage through the entire flow-through system verified that there was not a significant loss of sulfide with this apparatus (all n=7).

VII. Tissue homogenates.

Heart and lung tissues were kept on ice until use. Tissue was minced in Kreb’s buffer (mammalian) or Cortland buffer (piscine) on ice, and subsequently rinsed in
Figure 3. A trace from the AHSS comparing a single 10 μM Na₂S spike delivered by hand via syringe to a series five 1 μM spikes followed by a 5 μM spike delivered by the sulfidostat.
Figure 4. Schematic of the water-jacketed flow cell used for measurements of free H₂S in blood. A syringe withdrawal pump aspirated blood through the cell from sample syringe.
100mM potassium phosphate buffer or PEBBLE buffer 2 times and then homogenized on ice with an Ika-Werk Ultraturax Model SDT homogenizer with 10N probe. Homogenates were then spun in a Tomy micro centrifuge only long enough to reach 10k rpm, in order to remove unhomogenized tissue. Each batch of tissue was homogenized immediately prior to use, and a sample taken for protein assay.

VIII. Mitochondrial Isolation

Mitochondria from trout livers were obtained using the procedure described by Pallotti and Lenaz (Pallotti & Lenaz, 2007). Trout livers were removed from trout killed by a blow to the head and immediately placed in isolation buffer on ice. Livers were placed in fresh isolation buffer, finely minced, and rinsed 2 or 3 times with isolation buffer, with a final liver:buffer ratio of 1:10. The mince was homogenized on ice with an Ika-Werk Ultraturax Model SDT homogenizer with 10N probe intermittently until smooth, and then centrifuged at 750xg for 10 min. The supernatant was then filtered through cheesecloth and centrifuged at 13000 x g for 10 min, resuspended in isolation buffer and centrifuged again 10 min at 13000 x g. The final mitochondrial pellet was resuspended in a minimum volume of isolation buffer. Respiratory control (or coupling) ratios (RCR) were performed in trout RCR buffer (West & Driedzic, 1999) using succinate and ADP. In any experiments in which mitochondria were heat-killed, their container was held in 50°C water for 5 min.

Trout and steelhead heart mitochondria were isolated according to West and Driedzic (West & Driedzic, 1999). Hearts were minced in 9 volumes of trout heart
mitochondria isolation buffer on ice and homogenized with an Ika-Werk Ultraturax Model SDT homogenizer with 10N probe until smooth, and then centrifuged at 600g for 10 min and the supernatant spun at 9000g for 10 min. The pellet was resuspended in isolation buffer and spun again for 10 min at 9000g and resuspended in a minimum volume of isolation buffer. RCRs were performed as with trout liver mitochondria.

Bovine heart mitochondria were isolated based on our modifications of the procedure used in Dr. E.E. McKee’s lab (Mckee et al., 1990). 20 g of ventricle were placed in 200 ml MSE with 850 µl of Nargarse protease solution (Sigma) and minced on ice for 5 min. The mince was homogenized on ice intermittently with an Ika Ultraturax T18 Basic with an S18 N-19G probe until smooth. The homogenate was centrifuged at 1300 g for 5 min. The supernatant was vacuum filtered through 4 layers of cheesecloth and then spun at 14500 x g for 10 min. The pellet was rinsed with isolation buffer to remove the fluffy layer surrounding the mitochondrial pellet, and then resuspended in isolation buffer and spun again at 14500 x g for 10 min. The pellet was rinsed again with isolation medium and resuspended in a minimum volume of isolation buffer. RCRs were performed in mammalian RCR medium per Dr. McKee’s protocol, using glutamate and malate as substrates.

IX. Metabolism chamber.

A water-jacketed metabolism chamber was constructed for measurement of H₂S consumption. It consisted of a flat-bottom glass tube approximately 1.5 x 3 cm with an acrylic stopper machined to fit tightly; the latter with an injection port and a port for the
amperometric H₂S sensor and in some cases also an oxygen sensor. Samples were equilibrated by bubbling with the relevant gas mixture and then the stopper lowered until no air remained (final volume 1 ml or 1.5 ml) and homogenate filled the injection port to act as a diffusion buffer against O₂. The baseline(s) were then allowed to stabilize. Kreb’s buffer and red blood cell (RBC) ghosts were spiked with 10 µl of 1 mM Na₂S (10 µM final concentration) and allowed to incubate for at least 10 min. In the case of 5% bovine serum albumin (BSA) Krebs, incubation was allowed to continue until the sensor signal had returned to baseline, after which the sample was removed and assayed for sulfide with the diffusion chamber/methylene blue method as described below. This was done to determine whether the H₂S was reversibly binding to the BSA. Whole blood was spiked with 10 µl of 1 mM Na₂S sequentially.

Later experiments which used the Wosthoff gas mixing pump for finer control of oxygen levels were conducted in a homebuilt chamber which consisted of a lost-wax-cast soda lime glass inner member with O₂ and H₂S sensor ports on the side (Figure 5). Surrounding this was an acrylic jacket with water circulator and sensor ports. The stopper was made of polyvinylidene difluoride and sealed with a Viton o-ring. The stir bar was constructed from the core of a micro stir bar that had been removed from the Teflon shell, which has a very high oxygen memory, and inserted into a new shell of polyvinylidene difluoride and heat-sealed. This design was modeled after the Oroboros Oxygraph to minimize back-diffusion of O₂ from the chamber into the sample solution, and vice versa. After purging a sample of buffer for 10 min with N₂ and sealing the
Figure 5. Schematic of water-jacketed glass metabolism chamber. The stopper was made of polyvinylidene difluoride, as was the stir bar’s shell. The water outlet and second sensor port are not shown because they are out of the plane of the drawing. The second sensor port would be oriented perpendicular to the plane of the page. Filled black circle represent o-ring seals. s, stopper; bor, buna-N o-ring; aj, acrylic jacket; gc, glass case; sp, sensor port; sb, stir bar; vor, viton o-ring; wi, water in. Water out and two additional screws are out of the plane of the picture.
chamber, diffusion of O₂ into the chamber was measured to be 1.5±0.5 pmol sec⁻¹ (vs. ~7 for the acrylic chamber), which meets the standard reported for the Oroboros Oxygraph.

X. Colorimetric measurement of acid labile sulfide.

Assay of acid-labile sulfide was based on the method of Stipanuk and Beck (Stipanuk & Beck, 1982), modified by Geng et al. (Geng et al., 2004). Each diffusion chamber consisted of a 25 ml glass vial with three indentations around its wall that supported a polyethylene center well, cut out of a 5 ml test tube. A 3 cm x 3 cm piece of Whatman No. 1 filter paper was folded into quarters and placed in the center well such that no part of the paper touched the walls of the chamber. The vials had a screw cap with a Teflon seal. Zinc acetate (0.4 ml of 1%) was added to the filter paper to trap H₂S as ZnS, and two glass beads were added to the chamber to aid in mixing the sample. For plasma measurements the blood was divided into 3 aliquots immediately after removal from the animal. One aliquot of blood was spiked (spiked blood) with Na₂S (final concentration 10 µM, 15 µM for LW rat) and all three aliquots centrifuged for 3-5 minutes. Following centrifugation, 1 ml of untreated (control) plasma and 1 ml of plasma from the spiked blood (spiked blood) were added to the first and second chambers, respectively, immediately followed by 0.5 ml 50% w/v trichloroacetic acid (TCA), and quickly capped. The second aliquot of untreated plasma was then added to the third chamber, spiked to 10 µM (15 µM in the case of the LW rat) Na₂S (spiked plasma) and TCA added as above. The time between the addition of Na₂S to the blood and addition of TCA to the plasma was usually between 6 and 8 minutes and not more than 10
minutes. Chambers were incubated for 60 min on a shaker at 37°C (prior to incubation, chambers containing cow and pig plasma were at room temperature for ~1 h during transport from the slaughterhouse to the lab).

Following incubation, the center well with the filter paper and zinc acetate was removed and placed into a 5 ml glass vial containing 3.5 ml water. Then 0.4 ml of 20 mM N,N-dimethyl-\(p\)-phenylenediamine HCl in 7.2 M HCl was added, immediately followed by 0.5 ml of 30 mM FeCl\(_3\) in 1.2 M HCl, and the vial capped and gently inverted. Contents of the vials were transferred to 96-well plates and the absorbance read at 669 nm after 10 min. Absorbances were compared to those obtained from a Na\(_2\)S standard curve run in appropriate buffer in parallel with the experimental samples. This procedure recovered between 30 and 70% of the acid labile sulfide in a sample, based on comparison of standards to equivalent amounts of sulfide added directly to the filter paper. The lower detection limit for this assay was 1 µM.

XI. Sulfide-Ion Selective Electrode Measurement of Sulfide

Sulfide antioxidant buffer (SAOB) was made by dissolving 25 g sodium salicylate, 6.5 g ascorbic acid, and 8.5 g NaOH in water and diluting to 100 ml as per the instructions that accompanied the Lazar sulfide ISE. The Accumet silver/sulfide combination ion selective electrode (Fisher Scientific, Pittsburgh, PA) was immersed in a blank solution of 5 ml of sulfide antioxidant buffer (SAOB) and 10 ml Kreb’s buffer and the potential allowed to stabilize. The electrode was then transferred to a solution of 5 ml AOB and 10 ml Kreb’s buffer with 5% BSA that had been mixed immediately prior to
transfer. At approximately 1 h intervals the electrode was switched between the same blank and BSA solutions. The electrode was rinsed in a separate blank between transfers. The result was compared to a standard curve made by serial additions of 100 mM Na$_2$S to a 1:2 mixture of AOB and Kreb’s buffer following the manufacturer’s instructions. Later experiments with plasma were conducted with a 1:3 ratio of SAOB to plasma, which was the lowest ratio found in the literature (to give a conservative estimate of desulfuration). Continuous recording of the electrode potential was done with Biopac MP30 hardware and BSL Pro 3.7 software (Biopac Systems, Goleta, CA).

XII. PEBBLEs

PEBBLEs were produced using various modifications of the method described by Koo et al. (Koo et al., 2004). The following is a description of the most effective modification. Deviations from this are described in the results. In a round-bottom flask immersed in a water bath, 20 ml H$_2$O and 20 µl HNO$_3$ were heated to 60°C. 70µl phenyltrimethoxysilane dissolved in 200 µl EtOH were then added and allowed to hydrolyze for 27 min. 3 ml NH$_4$OH, 5 µl 10 mM sulforhodamine 101, and 15 µl 100 mM fluorescein mercuric acetate in 0.1 M NaOH (these were the stock concentrations of dye in all experiments) were then added quickly in that order. Once the solution was completely opaque (usually by 1 hr), 130 µl methyltrimethoxysilane in 200 µl EtOH were added and allowed to incubate until no further change in the solution was noted, usually 1 hr. The resulting pinkish-colored PEBBLEs were filtered through a 45 mm 0.1 µM MSI Magna Nylon filter, then resuspended with sonication in ~150 ml H$_2$O in the filter cup,
and filtered again without changing the filter membrane. Washes were repeated until the filtrate was nearly non-fluorescent visually (determined by looking into a cuvette in a fluorometer while illuminating with 490 nm light). The PEBBLEs were then dried on the filter at 50°C overnight. This procedure yielded ~50-60 mg of PEBBLEs, which were almost entirely non-adherent to the nylon filter membrane.

Initial experiments were conducted in the absence of cells to verify PEBBLE function. 200 µl of a 20 mg/ml suspension of PEBBLEs in 70/50 EtOH/H₂O were mixed with 0.75 ml H₂O and either 2.4 µl DTPA water (control for the Na₂S, which is made with DTPA water), 2.4 µl Na₂S, or 2.4 µl 1M cysteine. Mixtures were allowed to sit for 30 min with occasional shaking. They were then filtered and washed through 0.1 µm 25 mm MSI Magna Nylon filter membranes, and the trapped PEBBLEs resuspended in 0.5 ml PEBBLE buffer. 40 µl of the resuspended PEBBLEs were mixed with 200 µl mounting medium. The mounting medium was 2:1 glycerin:(bis)acrylamide, with the acrylamide being a solution of 540 mg acrylamide and 60 mg bis-acrylamide in 1 ml PEBBLE buffer. After vortexing, 10 µl of fresh 10% sodium bisulfite was added, the suspension vortexed again, and 10 µl placed on a slide, followed by a coverslip. Mounts were sealed with fingernail polish. In this method of mounting, the glycerin prevented the PEBBLEs from clumping and the polyacrylamide ensured that the PEBBLEs would stay stationary during imaging.

PEBBLEs were delivered to cells biolistically, using a ‘gene gun’. The gene gun was a paintball marker modified to accept a helium supply and using a home-
built PEBBLE cartridge holder. To load a cartridge, PEBBLEs were applied to an 8x8 cm piece of aluminum foil by laying the foil shiny-side down on the dried PEBBLEs and lightly pressing the middle of the foil with a blunt instrument such as a scalpel handle. The foil was then placed over the end of a PTFE cartridge, PEBBLEs towards the inside, and held in place by the retaining ring (Fig. 6). A small hole was made in the foil to allow it to tear during the shot. The cartridge was then inserted into the magazine, which was then inserted into the cartridge holder. To shoot cells, all the liquid was removed from the culture dish and the dish held vertically to allow as much medium as possible to drain from the coverslip. The helium pressure was set to 300 psi and the gene gun muzzle held ~ 3 cm away from the coverslip. After the shot, cells were rinsed with PBS immediately and either fixed or transferred to an incubator. Fixed cells were exposed to room air (control) or to 100 µM Na₂S for 15 min prior to mounting.

The appropriate normoxic or hypoxic 5% CO₂ environment for cells in the PEBBLE experiments was maintained in small plastic flip-top boxes connected to the outlet of bubbler-type humidifiers, which were connected to air/5% CO₂ and N₂/CO₂ tanks. The boxes and humidifiers were kept at 37° in a covered water bath, and the tubing leading to and from the humidifiers partially traveled through the water to ensure correct temperature and 100% humidity. The 12 mm coverslips on which breast cancer cells were growing were transferred from 12-well plates in which they were maintained to 35mm culture dishes containing DMEM/FBS with penicillin, streptomycin, and amphotericin B. The cells were taken to the lab and shot as described above, then rinsed with PBS. Fresh DMEM/FBS with antibiotics was added to the dishes, and they were
Figure 6. Gene gun and cartridge for loading PEBBLEs. PEBBLEs (p) were applied to an 8x8 cm piece of aluminum foil (f) by laying the foil shiny-side down on the dried PEBBLEs and lightly pressing the middle of the foil with a blunt instrument such as a scalpel handle. The foil was then placed over the end of a PTFE cartridge (pc). PEBBLEs towards the inside, and held in place by the retaining ring (rr).
placed in the incubator for 3 hours. At the end of the incubation the dishes were
removed, rinsed with PBS, and fixed in 2% paraformaldehyde/PBS for 6 min. Cells were
washed again, and incubated in 0.1% Triton-X100 for 10 min, followed by a rinse with
PBS and incubation for 10 min in To-Pro 3 to stain the nuclei. Coverslips were rinsed
with PBS, blotted, and mounted with Vectashield. Each treatment was performed in
triplicate.

XIII. Confocal Microscopy

Cells and isolated PEBBLEs were examined on a Zeiss LSM 710 laser-scanning
confocal microscope. Settings were: Plan-Apochromate 63x/1.40 Oil DIC M27
objective; beam splitter MBS 488/561/633; Channel 1 (FMA) 488nm Argon laser line at
15%, 492-586 filter, master gain 900, pinhole 51 µm; Channel 2 (sulforhodamine 101)
561nm diode laser at 3%, 565-635 nm filter, master gain 550, pinhole 57 µM; Channel 3
(ToPro-3) 633 nm HeNe laser line at 2%, 637-787 nm filter, master gain 500, pinhole 250
µM; Sequential frame scan, scan speed 9, averages 4, 12 bit recording. Cells were first
located through the oculars using the HBO 100 halogen lamp, keeping exposure time to a
minimum to avoid bleaching. Ten separate pictures were taken for each slide.

XIV. Analysis of confocal images

Histogram data for each image, with intensity bins ranging from 0 to 4095, were
saved as a text file and imported into Excel using a program written for the purpose in
SoftWire. If autofluorescence or excessive nuclear staining by ToPro-3 appeared bright
enough to overlap the relevant signal from the PEBBLEs, the selection tool in the Zeiss Zen software was used to manually select regions of the image which did not contain the problem areas, and the histogram data from only the selected areas used. In Excel, average intensity and area measurements were made for various intensity brackets. This corresponds in the Zen software to setting the intensity threshold values for a particular channel, which masks all pixels (in all three channels) below and above the minimum and maximum desired intensity. Typically, background fluorescence was not present beyond intensity bin 400, so beyond this most or all the fluorescence was from the PEBBLEs. Average bracketed intensity was calculated for each wavelength by multiplying the value of each intensity bin by the number of pixels in the bin, summing the product from each bin within the bracket, then dividing by the total number of bracketed pixels. Area was calculated simply as the total number of pixels in the bracketed intensity range. This was done for both the FMA and sulforhodamine signals, and the results reported as the ratio of the sulforhodamine to FMA signal. Area ratios tended to give more consistent results than intensity ratios, and results are reported based on area ratios. T-tests were performed using all thirty individual pictures for each treatment.

XV. Buffers

Krebs-Henseleit (mammalian; in mM): 115 NaCl, 2.5 KCl, 2.46 MgSO₄, 2 CaCl₂·2H₂O, 5.6 glucose, 1.38 NaH₂PO₄, and 25 NaHCO₃, pH 7.4. Cortland bicarbonate (fish; in mM): 124 NaCl, 74.6 KCl, 0.57 MgSO₄, 2 CaCl₂, Glucose 5.5, NaHCO₃ 12, NaH₂PO₄ 0.09, Na₂HPO₄ 1.8, pH 7.8. HEPES (trout in vivo; in mM): 145
NaCl, 3 KCl, 0.57 MgSO4, 2 CaCl2, 5 glucose, 3 HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) acid, 7 HEPES sodium salt (HEPES-Na), pH 7.8. Potassium phosphate: 100 mM KH2PO4/K2HPO4. Trout liver mitochondria isolation buffer: 250 mM sucrose, 24 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml fatty acid free BSA, pH 7.4. Trout heart mitochondria isolation buffer: 250 mM sucrose, 5 mM KH2PO4, 5 mM K2HPO4, and 2 mM EGTA, pH 7.4; Trout liver and heart mitochondria RCR buffer: 100 mM KCl, 12.5 mM KH2PO4, 12.5 mM K2HPO4, 10 mM Tris HCl, and 2.7 mg/ml fatty acid free BSA, pH 7.4; MSE: 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, and 2 mM EGTA, pH 7.0; Mammalian RCR buffer: 100 mM KCl, 50 mM MOPS, 5 mM K2HPO4, 1 mM EGTA, pH 7.4; PEBBLE/homogenization buffer: 120 mM KCl, 2 mM NaH2PO4, 3 mM Na2HPO4, 1 mM MgSO4, pH 7.0.

XVI. Iodometric Titration

Sulfide was determined by iodometric titration as described previously (American Public Health Association. et al., 1989). Briefly, an excess volume of 0.0250 M I2 in ~0.125 M KI, with ~1/10th the volume of 6 M HCl added, is reacted with the unknown sulfide sample, and the remaining I2 back-titrated with a 0.250 M solution of sodium thiosulfate using starch as an indicator. The thiosulfate solution is standardized against iodine formed from a standard potassium iodate solution upon addition of H2SO4.
XVII. Chemicals

Sodium sulfide nonahydrate crystals or sodium hydrosulfide hydrate flakes were placed in a glass syringe which was then sealed with a rubber stopper and sparged with N₂. N₂-bubbled distilled water containing 50 µM diethylenetriaminepentacetic acid (to chelate iron) was then injected into the syringe through the stopper to dissolve the Na₂S. Stock solutions were made fresh daily. This method was verified by iodometric titration (Clesceri et al., 1989). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO)

XVIII. Data Analysis

Data recorded on the Apollo were copied and pasted into Excel, where they were converted from sensor current to µM O₂ or H₂S. The converted data were saved as a .txt file and opened in Biopac for analysis. Half times for H₂S consumption were determined from exponential decay equations fit by Table Curve (Jandel, Chicago, IL). Statistical significance was determined by Students’ t-test or paired t-test, and correlation among groups was determined by a Spearman rank order correlation test using SigmaStat or SigmaPlot (Systat Software, San Jose, CA). Results are given as mean ± standard error or standard deviation; significance was assumed when p ≤ 0.05.
RESULTS

I. Amperometric H₂S Sensor Performance

Figure 7A represents part of an experiment comparing the commercial WPI sensor to my in-house version. In this experiment both sensors were installed in the same chamber and run simultaneously, ensuring identical environments. Over the course of 4 experiments consisting of a series of 5 µM or 0.1 µM spikes in pH 7.0 buffer, the WPI sensor averaged a signal-to-noise ratio of 1.58 ± 1.34%, while the home-built sensor averaged 0.35 ± 0.54%, calculated as the standard deviation of the signal noise at a plateau as a percent of the total signal. Figure 7B shows a standard curve from 0.5-25 µM at pH 7.4 in phosphate buffer. The lower limit of detection is ~20 nmol H₂S gas, or ~100 nmol total sulfide at pH 7.4 in saline.

II. Blood and Plasma Sulfide Measurement

II. A. i. Amperometric Measurements, Free Sulfide in Blood.

Free sulfide (H₂S gas corrected for temperature and pH; see methods) measured with the flow cell was not detected in trout, Lobund-Wistar (LW) and Harlan-Sprague-Dawley (HSD) rats, cow, pig, or C57/B6 mouse (Table 2). Once the baseline sulfide reading had stabilized, blood remaining in the sample syringe was spiked to 10 µM Na₂S, and measured sulfide did not exceed 2 µM. Figures 8 and 9 show flow cell traces from
Figure 7. In-house amperometric H₂S sensor performance. A: Comparison of in-house AHSS with commercial WPI, Inc sensor. The sensors were mounted in the acrylic chamber simultaneously and symmetrically such that they saw identical H₂S concentrations and the same stirring. A series of Na₂S spikes were added to increase the sulfide concentration in 1 µM increments, up to 10 µM. B: Standard curve done with homebuilt sensor. There is slight non-linearity at the higher concentrations but it is not relevant at typical experimental concentrations.
TABLE 2

WHOLE-BLOOD SULFIDE MEASURED WITH AMPEROMETRIC H₂S SENSOR

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trout (5)</td>
<td>ND</td>
<td>0.26 ±0.15</td>
</tr>
<tr>
<td>Mouse (5)</td>
<td>ND*</td>
<td>0.54 ±0.15</td>
</tr>
<tr>
<td>LW rat (5)</td>
<td>ND</td>
<td>0.42 ±0.07</td>
</tr>
<tr>
<td>HSD rat (3)</td>
<td>ND</td>
<td>NM</td>
</tr>
<tr>
<td>Pig (5)</td>
<td>ND</td>
<td>0.32 ±0.01</td>
</tr>
<tr>
<td>Cow (5)</td>
<td>ND</td>
<td>0.38 ±0.11</td>
</tr>
</tbody>
</table>

Note: Values are means ± SE; number of animals is in parentheses. Units are μM. ND, not detected; NM, not measured; LW, Lobund-Wistar; HSD, Harlan Sprague Dawley. Less than 10s elapsed between spiking the sample and measurement. *Sensor response was atypical, slowly rising to 0.22 ± 0.02. This response is not characteristic of free sulfide.
Figure 8. Flow cell trace of trout blood showing absence of baseline sulfide and response to 10 µM Na₂S spike. Blood, point at which blood reached the sensor; spiked blood, point at which spiked blood reached sensor; wash, point at which buffer reached sensor. Inset: Flow cell trace of buffer spiked to 10 µM Na₂S. w, wash.
Figure 9. Flow cell trace of rat blood showing absence of baseline sulfide and response to 10 µM Na₂S spike. Blood, point at which blood reached the sensor; spiked blood, point at which spiked blood reached sensor; wash, point at which buffer reached sensor.
washed blood

spiked blood

blood

Total Sulfide (µM)

time (min)
trout and rat blood, respectively. Blood from other mammals was similar to rat blood (Whitfield et al., 2008).

II.A. ii. Amperometric Measurements, Sulfide Metabolism

Figures 10, 11, and 12A show traces of 10 µM sulfide spikes to trout blood, rat blood and BSA, respectively, in the metabolism chamber (Whitfield et al., 2008). Table 3 shows half-times for whole blood sulfide consumption in the various animals tested. Blood from all animals tested consumed sulfide, as did bovine plasma and BSA. Sulfide concentrations decayed exponentially to baseline. Sulfide consumption by BAS was dependent on BSA concentration and was essentially nil in protein free solutions (Figure 12B). The half-time for decay of a 10 µM Na₂S spike in buffer following addition of an excess of zinc acetate, which very rapidly removes sulfide from solution, was 23.7 ± 0.6 s at 15°C and 13.0 ± 0.2 s at 37°C (all n=4). Red blood cell ghosts had no effect on sulfide consumption or sensor response (not shown). Figure 12B shows the O₂ dependence of BSA-mediated sulfide consumption.

II.B. Colorimetric Measurement.

Acid-labile sulfide values in plasma are shown in Table 4 (Whitfield et al., 2008). Control plasma was ~2 µM in lamprey, trout, cow, and pig, not detected in LW rat, and 4.3 µM in HSD rat. Acid-labile sulfide in plasma isolated from spiked whole blood had returned to control levels by the time of assay. Recovery for the spiked plasma averaged 79 ± 21% when the calculation presumes the spike is additive to the measured baseline for each sample, and were near 101±13% if the baseline is not included. Acid labile
Figure 10. Consumption of exogenous sulfide by whole trout blood measured in the acrylic metabolism chamber. Each spike in blood represents enough Na₂S to raise total sulfide to 10 μM in the absence of consumption.
Figure 11. Consumption of exogenous sulfide by whole rat blood measured in the acrylic metabolism chamber. Each spike in blood represents enough Na$_2$S to raise total sulfide to 10 μM in the absence of consumption.
Figure 12. Consumption of sulfide by bovine serum albumin measured with the amperometric H$_2$S sensor in the acrylic metabolism chamber. A. Trace of 10 µM Na$_2$S spike in 5% BSA Kreb’s buffer. B. Dependence of BSA concentration and oxygen level on sulfide consumption. At each [BSA] there was no difference between normoxic and anoxic rates (p>0.05). There was no difference in rate between 0 and 0.1% BSA for anoxic or normoxic. 0 vs. 1% and 0 vs. 5% were significantly different for anoxia and normoxia. n=3
A

B

Sulfide Consumption (µM min⁻¹)

0.0 0.4 0.8 1.2 1.6 2.0

% BSA

0 0.1 1.0

normoxic
anoxic

0 5 10 15 20

time (min)

µM sulfide

0 10 20

0 5 10 15 20

time (min)
### TABLE 3

HALFTIMES OF SULFIDE CONSUMPTION BY WHOLE BLOOD, PLASMA, AND 5% BSA

<table>
<thead>
<tr>
<th></th>
<th>Half time, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamprey (4)</td>
<td>15.0 ± 3.1</td>
</tr>
<tr>
<td>Trout (6)</td>
<td>12.7 ± 3.2</td>
</tr>
<tr>
<td>L-W rat (3)</td>
<td>130.5 ± 22.8</td>
</tr>
<tr>
<td>H-S-D rat (3)</td>
<td>151.1 ± 8.2</td>
</tr>
<tr>
<td>Pig (3)</td>
<td>76.0 ± 12.9</td>
</tr>
<tr>
<td>Cow (3)</td>
<td>51.3 ± 13.3</td>
</tr>
<tr>
<td>Cow plasma (3)</td>
<td>300 ± 30</td>
</tr>
<tr>
<td>5% BSA anoxic (3)</td>
<td>216 ± 38</td>
</tr>
<tr>
<td>5% BSA normoxic (7)</td>
<td>191 ± 17</td>
</tr>
</tbody>
</table>

Note: Values are means ± SE; number of animals is in parentheses; BSA was dissolved in Kreb’s buffer
TABLE 4
ACID-LABILE SULFIDE CONCENTRATION IN PLASMA OF VARIOUS ANIMALS ASSAYED WITH THE INDIRECT METHYLENE BLUE METHOD

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Spiked Blood</th>
<th>Spiked Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamprey</td>
<td>2.6 ±0.6</td>
<td>1.2 ±1.0</td>
<td>10.2 ±0.6 (76%, 102%)</td>
</tr>
<tr>
<td>Trout</td>
<td>1.6 ±0.5</td>
<td>2.0 ±0.1</td>
<td>10.8 ±0.5 (92%, 108%)</td>
</tr>
<tr>
<td>Trout overnight</td>
<td>1.3±0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LW rat</td>
<td>ND</td>
<td>ND</td>
<td>15.6 ±1.0 (104%)</td>
</tr>
<tr>
<td>HSD rat</td>
<td>4.3 ±0.5</td>
<td>NM</td>
<td>10.4 ±0.7 (61%, 104)</td>
</tr>
<tr>
<td>Pig</td>
<td>2.3 ±0.2</td>
<td>1.5 ±0.1</td>
<td>8.6 ±0.2 (63%, 86%)</td>
</tr>
<tr>
<td>Cow</td>
<td>2.1 ±0.4</td>
<td>1.1 ±0.1</td>
<td>9.4 ±0.8 (73%, 94%)</td>
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</tbody>
</table>

Values are means ± SE; n=6, HSD rat; n=8, trout; n=4, all others. Units are µM. Plasma, measurement of sulfide in unaltered plasma; Spiked Blood, plasma obtained from whole blood spiked with Na₂S (final concentration 10 µM, 15µM in LW rat) prior to separation; Spiked Plasma, plasma spiked with Na₂S (final concentration 10 µM, 15µM in LW rat) after centrifugation. First number in parentheses shows % recovery of Na₂S spike accounting for measured baseline sulfide, second number shows % recovery assuming zero baseline sulfide. LW; Lobund Wistar; HSD, Harlan Sprague Dawley; ND, not detected; NM, not measured.
sulfide in trout red blood cells and plasma that had been kept at 4°C for one day was
2.33±0.26 µM and 1.28±0.18 µM, respectively. In a different experiment, 5% BSA
Kreb’s buffer was spiked to 10 µM Na₂S in the metabolism chamber and allowed to
incubate until the amperometric H₂S sensor reading had returned to baseline. When that
buffer was assayed with the methylene blue method it contained 2.8 ±0.3 µM sulfide, not
significantly different from an unspiked control (3.3 ±0.6 µM; n=4).

II.C. Sulfide Ion-Selective Electrode.

Sulfide was liberated from 5% BSA upon mixing with sulfide alkaline antioxidant
buffer (SAOB) and reached levels above 1 mM by 12 hr, as seen in Figure 13 (Whitfield
et al., 2008). In Lamprey plasma sulfide reached a level of 15 µM (Fig. 14), in trout
plasma the sulfide level at 2.5 hr had reached 34±10 µM (Fig. 15), and at 2 hr
mammalian (bovine and ovine) plasma had reached 18±8 µM (Fig. 16). Acidification
after 12 hrs produced the smell of H₂S. Transfer of the ISE to a blank caused the reading
to drop to near the baseline, and returning the ISE to the sample resulted in a return of the
reading to the expected level, indicating the apparent increase in sulfide was not sensor
drift. An indirect methylene blue assay of the bovine and ovine plasma after 2 days in
SAOB found 1664 ± 141 µM whereas control plasma from the same animals (kept in the
refrigerator 2 days, SAOB added immediately prior to the assay), showed a level of 3.8 ±
1 µM. Figure 17 shows the effect of spiking blood with 10 µM H₂S while measuring
with the ISE.
Figure 13. Sulfide production from bovine serum albumin in sulfide antioxidant buffer measured with the sulfide ion selective electrode. Dotted line shows stability of a 30 µM spike in Kreb’s buffer/SAOB.
Figure 14. Sulfide production from lamprey plasma in antioxidant buffer measured with the ISE. A: Trace of sulfide production. b, ISE into a blank; e, ISE back into experimental. B: Average sulfide production, n=3 30, 60, 90, time in min; pb, pre-blank; b, blank; pob, post-blank.
Figure 15 Sulfide production from trout plasma in antioxidant buffer measured with the ISE. A: Trace of sulfide production. b, ISE into a blank; e, ISE back into experimental. B: Average sulfide production, n=3
Figure 16 Sulfide production from mammalian plasma in antioxidant buffer measured with the ISE. A: Trace of sulfide production in bovine plasma. b, ISE into a blank; e, ISE back into experimental. B: Average sulfide production, n=1 bovine, 2 ovine. b, blank.
Figure 17. Response of the ISE to a 10 µM Na₂S spike in bovine plasma. Sample was spiked to 10 µM Na₂S ~5 min after mixing with SAOB.
III.A Tissue Production and Consumption

III.A.i. Trout Gill

Homogenized trout gill arches produced sulfide from cysteine, measured in the diffusion chamber (Olson et al., 2008). Baseline sulfide content was measured by adding TCA at time zero, and averaged 0.17±0.02 nmol mg protein⁻¹. When samples were corrected for baseline sulfide content, production was 1.05 ± 0.3 pmol min⁻¹ mg protein⁻¹. With the H₂S-synthesis inhibitors AOAA and PPG present, production was decreased to 0.18 pmol min⁻¹ mg protein⁻¹ (p=0.003)

III.A. ii. Steelhead Heart

Minced steelhead heart measured in the metabolism chamber produced H₂S from cysteine (Fig. 18). At 100 µM cysteine, sulfide production was 5.9 ± 7.8 pmol min⁻¹ g tissue⁻¹, however this measurement likely represents sensor drift. At 1 mM cysteine, sulfide production was 47.1 ± 7.5 pmol min⁻¹ g tissue⁻¹ and increased to 88.1 ± 25.7 pmol min⁻¹ g tissue⁻¹ when cysteine was 10 mM. When given 220 nmol O₂ (amount sufficient to raise chamber to 150 µM) sulfide consumption dominated, at a rate of 856 ± 256 pmol min⁻¹ g tissue⁻¹. Upon depletion of oxygen (presumably; O₂ was not measured during this experiment) consumption resumed at a faster rate, although the increase was not significant. In steelhead ventricle homogenate which had been centrifuged at 9000g to remove the mitochondria, anoxic production was observed when given 10 mM cysteine (18.1 ± 1.3 pmol min⁻¹ mg protein⁻¹). When normoxic, no production was observed (0.96 pmol min⁻¹ mg protein⁻¹). After measuring production in normoxia, the homogenate
Figure 18 Sulfide production from steelhead heart homogenate in acrylic metabolism chamber. A: Trace from the AHSS showing sulfide production from 0.1, 1 and 10 mM cysteine. The sulfide is consumed upon addition of a bubble of oxygen. B: Average sulfide production at different concentrations of cysteine. Rates at 1 (*) and 10 mM (**) were significantly greater than at 0.1 mM. p=0.01 and 0.02. n=3.
was bubbled with N₂, after which, with no additional cysteine added, production was
9.48 ± 3.15 pmol min⁻¹ mg protein⁻¹.

III.A.iii. Bovine Heart

Bovine ventricle homogenate in the metabolism chamber produced sulfide from 1 mM cysteine (Fig. 19, 65.9 ± 8.4 pmol sulfide min⁻¹ mg protein⁻¹). When given 25 nmol O₂ (sufficient to raise the chamber O₂ concentration to 15 µM) consumption occurred at a rate of 78.3 ± 34.4 pmol min⁻¹ mg protein⁻¹. Upon depletion of the oxygen production resumed at 85.0 ± 25.7 pmol min⁻¹ mg protein⁻¹.

III.A. iv. Bovine, Seal, and Rat Lung Homogenate

Sulfide production was stimulated in anoxic bovine lung homogenate by addition of two 5 µM Na₂S spikes and 1 mM reduced glutathione. When anoxic, this preparation produced sulfide at a rate of 5.2 ± 2.5 pmol min⁻¹ mg protein⁻¹. In this setup anoxia was maintained by keeping N₂ in the injection port of the chamber stopper, which was not filled with liquid as is usually the case, and thus gas in the port had access to the bulk solution. When the N₂ was removed, air was then able to diffuse into the solution at a rate sufficient to reverse the sulfide production and cause net consumption at a rate of 46.4 ± 11.5 pmol min⁻¹ mg protein⁻¹. Sulfide production and consumption were easily reversible by removing the source of N₂ from the port (Fig. 20).

After a series of four 5 µM additions to bovine lung homogenate at near-anoxia H₂S consumption was 18.2 ± 6.6 pmol sulfide min⁻¹ mg protein⁻¹ (Fig. 21). Upon addition of 1 mM reduced glutathione the average consumption rate decreased to 1.0 ± 2.01 pmol sulfide min⁻¹ mg protein⁻¹ (p = 0.02).
Figure 19. Bovine ventricle homogenate sulfide production measured in acrylic metabolism chamber. A: Trace from the AHSS showing production of sulfide following addition of 1 mM cysteine (100 µM PLP had been added previously). Upon addition of 25 nmol of O₂ in the form of an air bubble net consumption dominated until most of the oxygen was consumed, after which production again dominated. Black line, sulfide; Gray line, oxygen. B: Average sulfide production and consumption. Production rate in the presence of cysteine was significantly higher than that in the absence of cysteine (*). PLP, prior to cysteine; air, after injection of air bubble; recovery, production following disappearance of oxygen. n=3
Figure 20. Bovine lung homogenate sulfide production and consumption measured in acrylic metabolism chamber after addition of Na$_2$S and 1 mM glutathione. After the second 5 µM Na$_2$S spike a brief period of sulfide consumption was followed by a gradual production that was reversed by addition of air. Switching between N$_2$ or air in the stopper port allowed transitioning between production or consumption. A: Trace from the AHSS. GSH, 1 mM reduced glutathione; S, two additions of 5 µM sulfide; air, removal of N$_2$ flow from port; N$_2$, N$_2$ present in port. Subsequent increases and decreases in sulfide are more N$_2$/air transitions. B: Average rates of consumption and production, n=3.
Figure 21. Bovine lung homogenate consumption and production of sulfide measured in acrylic metabolism chamber after addition of 5 µM spikes of Na$_2$S and single additions of 1 mM GSH or GSSG. A: Traces from the AHSS showing increase in the rate of sulfide consumption with addition of oxidized glutathione (GSSG) and decrease in the rate with addition of reduced glutathione (GSH), as well as the very rapid sulfide consumption upon addition of air to chamber regardless of the form of glutathione. Black line, sulfide; Gray line, oxygen. B: Average consumption rates before and after addition of GSH and GSSG. Rates of consumption prior to addition of glutathione were not different between experiments. Consumption rate in the presence of GHS is significantly slower than in its absence (*, p=0.02). Consumption rate after addition of GSSG is significantly more rapid than in its absence (**, p<0.004). n=3
In a similar experiment initial consumption was 21.7 ± 7.7 pmol sulfide min⁻¹ mg protein⁻¹ (p=0.71 vs. initial rate of previous experiment), and upon addition of 1 mM oxidized glutathione the consumption rate increased to 88.9 ± 18.4 pmol sulfide min⁻¹ mg protein⁻¹ (p=0.004). In all cases addition of 1.5 µl of air caused an extremely rapid consumption.

Homogenized bovine lung tissue was not observed to produce H₂S in the presence of 100 µM pyridoxal phosphate (PLP) or PLP + 1 mM cysteine, but produced H₂S at a rate of 5.6 ± 3.9 pmol H₂S min⁻¹ mg protein⁻¹ immediately once 1 mM α-ketoglutarate had been added in addition to cysteine and PLP (Fig. 22; Olson et al. 2010). Consumption in the presence of 12.5 nmol O₂ was 100 ± 83 pmol H₂S min⁻¹ mg protein⁻¹, and upon depletion of O₂ production resumed at a rate of 13 ± 9 pmol H₂S min⁻¹ mg protein⁻¹. When given α-ketoglutarate before cysteine, no H₂S production was observed until the cysteine was added, and there was a delay of between 1 and 5 min after cysteine addition before production was measurable (Fig. 23). Sulfide was produced at a rate of 30.8 ± 8.4 pmol min⁻¹ mg protein⁻¹ initially, consumed at 492± 75 pmol min⁻¹ mg protein⁻¹ upon addition of O₂, and resumed at a rate of 80 ± 12 pmol min⁻¹ mg protein⁻¹ upon depletion of O₂. When given 1 mM succinate following addition of 1 mM cysteine, the pattern of production and consumption was similar to α-ketoglutarate (Fig. 24). Production after succinate addition began immediately and was 13.7 ± 7.9 pmol min⁻¹ mg protein⁻¹, followed by consumption at a rate of 178 ± 92 pmol min⁻¹ mg protein⁻¹, and recovery after oxygen at 34.1± 17.3 pmol min⁻¹ mg protein⁻¹.
Figure 22. Bovine lung homogenate sulfide production from cysteine after $\alpha$-ketoglutarate addition, measured in glass metabolism chamber. A: Trace from the AHSS showing sulfide production after addition of 1 mM cysteine followed by 1 mM $\alpha$-ketoglutarate (100 µM PLP added previously). When 12.5 nmol O$_2$ was added as a bubble of air, consumption dominated until the O$_2$ had been consumed. Black line, sulfide; Gray line, oxygen. B: Average production and consumption rates. The rate of production was significantly higher after the addition of cysteine ($p=0.02$, *, **) $n=5$. 
**Figure 1:**

Panel A: Graph showing the recovery of H_2S in pmol min^(-1) mg protein^(-1) with time in minutes, with air and cysteine (cys) supplementation.

Panel B: Graph indicating the effect of a-kg treatment on the recovery of H_2S.
Figure 23. Bovine lung homogenate sulfide production from cysteine, added following \(\alpha\)-ketoglutarate addition, measured in glass metabolism chamber. A: Trace from AHSS showing sulfide production after addition 1 mM \(\alpha\)-ketoglutarate followed by 1 mM cysteine (100 \(\mu\)M PLP added previously). The lag following addition of cysteine, which was not present when cysteine was added before \(\alpha\)-kg, was characteristic of cysteine addition after \(\alpha\)-kg. When 12.5 nmol O\(_2\) was added as a bubble of air, consumption dominated until the O\(_2\) had been almost completely consumed. Black line, sulfide; Gray line, oxygen. B: Average production and consumption rates. Each average is significantly different from the others (P<0.01, *). n = 3.
A) Graph showing µM sulfide and O2 over time (min) with markers for a-kg and cys.

B) Bar graph showing pmol sulfide min⁻¹ mg protein⁻¹ with treatments for 1 mM akg, 1 mM cys, 12.5 nmol O₂, and recovery, marked with asterisks indicating significant differences.
Figure 24. Bovine lung homogenate sulfide production from cysteine after addition of succinate, measured in glass metabolism chamber. A: Trace from the AHSS showing sulfide production after addition of 1 mM cysteine followed by 1 mM succinate (100 µM PLP added previously). When 12.5 nmol O₂ was added as a bubble of air, consumption dominated until the O₂ had been almost completely consumed. Black line, sulfide; Gray line, oxygen. B: Average production and consumption rates. Each average is significantly different from the others (p<0.05, *). n=5.
A

µM sulfide and O₂

time (min)

0 1 2 3 0

µM sulfide and O₂

air

cys

succ

B

pmol sulfide min⁻¹ mg protein⁻¹

1 mM cys

1 mM succ

12.5 nmol O₂

recovery

*
When bovine lung homogenate H\textsubscript{2}S consumption (2 \textmu M Na\textsubscript{2}S spikes) was examined at various oxygen levels, H\textsubscript{2}S consumption was constant at \(\sim 220\) pmol min\(^{-1}\) mg protein\(^{-1}\) at 20 and 40 \mu M O\textsubscript{2} and began falling at \(\sim 10\) \mu M, to a minimum of \(18.7 \pm 3.1\) pmol min\(^{-1}\) mg protein\(^{-1}\). Oxygen consumption during the same period was 540\(\pm 40\) pmol min\(^{-1}\) mg protein\(^{-1}\) at 40 \mu M O\textsubscript{2} and continuously dropped to a minimum of 15.3 \pm 2.5 pmol min\(^{-1}\) mg protein\(^{-1}\) at zero oxygen (Fig. 25).

III.A.v. Sulfide production by Seal lung

In the H\textsubscript{2}S sensor chamber, anoxic homogenates of lung tissue from seal (Fig. 26) produced H\textsubscript{2}S when given PLP, cysteine, and \(\alpha\)-ketoglutarate, at a rate of 4.3\(\pm 1.7\) pmol H\textsubscript{2}S min\(^{-1}\) mg protein\(^{-1}\), while in the presence of 12.5 nmol O\textsubscript{2} (given as 1.5 \mu l room air) consumed the previously produced H\textsubscript{2}S at a rate of 24.0 \pm 10.2 pmol H\textsubscript{2}S min\(^{-1}\) mg protein\(^{-1}\) (Olson et al., 2010). Once the O\textsubscript{2} was consumed production resumed at a rate of 14.1 \pm 7.4 pmol H\textsubscript{2}S min\(^{-1}\) mg protein\(^{-1}\).

Rat lung homogenate produced H\textsubscript{2}S in a pattern similar to that of bovine and seal tissue (Fig. 27). When provided with 1 mM cysteine, H\textsubscript{2}S was produced at a rate of 1.8 \pm 0.8 pmol min\(^{-1}\) mg protein\(^{-1}\). After addition of 1 mM \(\alpha\)-ketoglutarate production increased to 8.8 \pm 1.0 pmol min\(^{-1}\) mg protein\(^{-1}\) (p=0.006). Upon the addition of 1.5 \mu l air (12.5 nmol O\textsubscript{2}, sufficient to raise [O\textsubscript{2}] to 8.3 \mu M if none was consumed) sulfide consumption occurred at a rate of 95.0 \pm 27 pmol min\(^{-1}\) mg protein\(^{-1}\). Once all the O\textsubscript{2} was depleted, production resumed at faster rate than before the air (18.5 \pm 1.4 pmol min\(^{-1}\) mg protein\(^{-1}\), p=0.005).
Figure 25. Bovine lung homogenate sulfide and oxygen consumption at various oxygen concentrations, measured in glass metabolism chamber. Homogenate was bubbled with various air/N₂ mixtures in the metabolism chamber, then sealed and spiked to 2 µM Na₂S. Open squares, oxygen; closed circles, sulfide. O₂ and H₂S consumption rates were significantly different from each other at 40, 20, 7.3, and 5.9 µM O₂. H₂S consumption was significantly greater at 40 µM O₂ than at 5.9 µM O₂ and below. n=3
Figure 26. Seal lung homogenate sulfide production from cysteine after addition of α-ketoglutarate, measured in glass metabolism chamber. A: Trace from the AHSS showing sulfide production after addition of 1 mM cysteine followed by 1 mM α-ketoglutarate (100 µM PLP added previously). When 12.5 nmol O₂ was added as a bubble of air, consumption dominated until the O₂ had been almost completely consumed. Black line, sulfide; Gray line, oxygen. B: Average production and consumption rates. Rate of production after akg (*) and recovery (**) was greater than in the presence of cys alone. Rate of production during recovery was not significantly different than after akg. n=3.
Figure 27. Rat lung homogenate sulfide production from cysteine after α-ketoglutarate addition, measured in glass metabolism chamber. A: Trace from the AHSS showing sulfide production after addition of 1 mM cysteine followed by 1 mM α-ketoglutarate (100 µM PLP added previously). When 12.5 nmol O₂ was added as a bubble of air, consumption dominated until the O₂ had been almost completely consumed. The steady decrease in the oxygen trace is sensor drift. Black line, sulfide; Gray line, oxygen. B: Average production and consumption rates. p=0.006, *; p=0.005, **, relative to baseline. n=3.
III.B. Mitochondrial Consumption

III.B. i. Steelhead Heart

Steelhead heart mitochondria consumed a 5 µM Na2S spike approximately twice as fast in air-equilibrated buffer as in anoxic buffer (Fig. 28, 30.5±2.6 vs. 15.5±2 nmol sec⁻¹ mg protein⁻¹, p=0.003). Heat-killed mitochondria did not consume sulfide significantly slower than anoxic (11.4±3.8 nmol sec⁻¹ mg protein⁻¹, p=0.32).

III.B.ii. Steelhead Liver

Isolated trout liver mitochondria consumed H₂S in an oxygen-dependent manner as well (Fig. 29). Using the sulfidostat to measure oxygen and H₂S consumption at constant H₂S levels, it was determined that 1.45±0.14 molecules of O₂ were consumed per H₂S molecule, independent of H₂S concentration (Fig. 30). It was also shown that the rate of consumption was inversely dependent on the concentration of H₂S, with the maximum rate occurring at 0.5 µM (3.0 ± 0.8 nmol min⁻¹ mg protein⁻¹) and the minimum rate at 15 µM (1.25 ± 0.26 nmol min⁻¹ mg protein⁻¹, Fig. 31). It was also shown that in all cases the average consumption rate was less at the end of each run (after ~7 min sulfide exposure) than at the beginning, although this was only significant at 15 µM and 20 µM (p=0.046 and 0.03 respectively) and there was not an obvious pattern to which concentrations resulted in a larger decrease.

III.B. iii. Bovine Heart

In bovine heart mitochondria at ~130 µM O₂ (roughly arterial), also using the sulfidostat, the stoichiometry was 1.11±0.06 molecule of O₂ per H₂S molecule, also independent of H₂S concentration (Fig. 32). Bovine heart mitochondria did not show
Figure 28. Steelhead heart mitochondria sulfide consumption, measured in acrylic metabolism chamber. Mitochondria were equilibrated to either room air or N₂, the chamber sealed, and then spiked to 5 μM Na₂S. Mitochondria were heat-killed by immersion in 50°C water for 10 min. Anoxic mitochondria did not consume sulfide faster than heat-killed. Normoxic mitochondria consumed sulfide significantly faster than anoxic (*) or head-killed (**). n=4
**Bar chart showing nmol sulfide min⁻¹ mg protein⁻¹ for normoxic, anoxic, and heat-killed conditions.**

- Normoxic: High concentration, indicated by a large bar.
- Anoxic: Moderate concentration, indicated by a medium-sized bar.
- Heat-killed: Low concentration, indicated by a small bar.

Significance levels: ***, *", indicating statistical significance. ** Indicates a higher significance level than a *.
Figure 29. Traces of trout liver mitochondria consumption of oxygen and sulfide while using the sulfidostat in acrylic metabolism chamber. Once equilibrated to room air in the metabolism chamber, the chamber was sealed and spiked to the desired sulfide concentration. The sulfidostat was then set to maintain sensor current at the plateau level for each concentration.
Figure 30. Ratio of oxygen to sulfide consumed by steelhead liver mitochondria at various sulfide concentrations maintained using the sulfidostat. There was no significant trend in the data. n=4
Figure 31. Rate of sulfide consumption by steelhead liver mitochondria as a function of sulfide concentration. Black circles are initial rates, gray squares are final rates (after ~5 min at a given sulfide level maintained by the sulfidostat in the acrylic metabolism chamber). Initial and final rates were only different from each other at 15 and 20 µM sulfide. Initial slope = -0.069±0.017, $R^2$ of 0.61. Final slope = 0.093±0.01, $R^2$ of 0.91. n=4
Figure 32. Ratio of oxygen to sulfide consumption for bovine heart mitochondria at various sulfide concentrations, using the sulfidostat in the glass metabolism chamber. n=4
the clear progressive inhibition of consumption that was seen in trout liver mitochondria. The bovine mitochondria, when measured within the first minute of consumption, consumed sulfide more quickly at higher sulfide concentrations up to approximately 7 µM, at which point the consumption rates leveled off. The sulfide consumption rate at 0.5 µM Na₂S was 12.0 ± 1.6 nmol min⁻¹ mg protein⁻¹, while at 20 µM Na₂S the rate had increased to 24.5 ± 10 nmol min⁻¹ mg protein⁻¹ (p=0.02). After ~5 minutes, however, increased inhibition was evident from 7 µM to 20 µM, but not at lower concentrations (Fig. 33). The rate after 5 min at 0.5 µM sulfide was 8.7 ± 0.6 nmol min⁻¹ mg protein⁻¹ while the rate at 20 µM was 15.2 ± 6 nmol min⁻¹ mg protein⁻¹. None of the rate changes were significant, however.

When examining H₂S consumption at lower oxygen concentrations, the consumption rate was steady at ~8 nmol min⁻¹ mg protein⁻¹ from 40 µM to 1.9 µM O₂ and abruptly dropped to 0.4 ± 0.1 nmol min⁻¹ mg protein⁻¹ at 0 oxygen (Fig. 34). Oxygen consumption during the same period was steady at ~22 nmol min⁻¹ mg protein⁻¹ from 40 µM to 5.9 µM O₂, after which consumption began to drop, reaching a minimum of 0.5 ± 0.1 nmol min⁻¹ mg protein⁻¹ at zero oxygen. At 40 µM O₂ in the presence of electron transport chain inhibitors H₂S consumption was inhibited to various degrees (Fig. 35). Rotenone and 3-nitropropionic acid (inhibit NADPH oxidase and succinate dehydrogenase, respectively) did not significantly inhibit consumption (7.2 ± 1.4, p=0.37 and 5.8 ± 1.2, p=0.14, respectively vs. control of 7.8 ± 1.4 nmol min⁻¹ mg protein⁻¹), while the complex IV inhibitor azide (0.6 ± 0.2, p<0.001) and complex III inhibitor antimycin A (1.4 ± 0.4, p=0.001) significantly inhibited consumption vs. control.
Figure 33. Bovine heart mitochondria sulfide consumption rate as a function of sulfide concentration using sulfidostat in the glass metabolism chamber. Black circles are initial rate, gray squares are final rate (after ~5 min at a given sulfide level maintained by the sulfidostat). The only significant difference was between rates at 20 µM (p=0.02). n=4
µM sulfide

nmol sulfide min⁻¹ mg protein⁻¹

0 5 10 15 20 25

0 5 10 15 20 25

40 35 30 25 20 15 10 5
Figure 34. Bovine heart mitochondria sulfide and oxygen consumption rates as a function of initial oxygen concentration, measured in the glass metabolism chamber. Buffer was bubbled with various air/N₂ mixtures, and when equilibrated the chamber was sealed and spiked to 2 µM Na₂S. Once the sulfide reading stabilized the mitochondria were injected. Open squares, oxygen; closed circles, sulfide. n=6
Figure 35. Bovine heart mitochondria sulfide consumption in the presence of electron transport chain inhibitors, measured in the glass metabolism chamber. Buffer with various inhibitors was equilibrated to 40 µM O₂, the metabolism chamber sealed, and spiked to 2 µM Na₂S. The mitochondria were injected once the sulfide reading stabilized. Azide, and complex IV inhibitor, and antimycin A, a complex III inhibitor, significantly inhibited consumption vs control (p<0.001 and p=0.001, respectively). n=6
III.C. Bovine Pulmonary Artery Smooth Muscle Cell Production and Consumption.

Bovine pulmonary artery smooth muscle cells (PASMC) consumed sulfide oxygen-dependently (Fig. 36). When given a spike of 2 µM Na₂S, PASMC consumed the Na₂S at 2.80 ± 1.3 and 2.66 ± 1.0 pmol 1k cells⁻¹ min⁻¹ in 127 µM and 39.9 µM O₂, respectively, and the difference is not statistically significant (p=0.8). These O₂ values correspond to arterial and venous [O₂]. Consumption rate decreased progressively at 7.5 µM O₂ (0.93 ± 0.43 pmol 1k cells⁻¹ min⁻¹), 1.9 µM O₂ (0.22 ± 0.15 pmol 1k cells⁻¹ min⁻¹), and 0 µM O₂ (0.08 ± 0.07 pmol 1k cells⁻¹ min⁻¹). The consumption rates at these three oxygen levels are all significantly different from each other and from the higher levels (p = 0.03 for 1.9 µM vs. 0 µM, p<0.001 all others). These three lower oxygen concentrations correspond to the low-micromolar intracellular oxygen levels typically reported (Jones & Kennedy, 1982; Erecinska & Silver, 2001). Consumption was 2.09 ± 0.75 pmol 1k cells⁻¹ min⁻¹ when cells were tested with 127 µM at the end of each experiment, which is not significantly different from either the first 127 µM or 39.9 µM. Bovine PASMC were not observed to produce H₂S from cysteine with or without addition of α-ketoglutarate.
Figure 36. Bovine pulmonary artery smooth muscle cell consumption of sulfide at various oxygen levels, measured in the glass metabolism chamber. Cell suspensions were equilibrated at various air/N₂ ratios in the metabolism chamber, then sealed and spiked to 2 µM Na₂S. Experiments on a given batch of cells always started and ended at 127 or 40 µM O₂, with the middle O₂ concentrations in random order. Consumption rates at 7.5, 1.9, and 0 µM O₂ are significantly different from each other and from the higher levels. n= 4 for 127 µM end; n=5 for 40 µM end; n=6 for 1.9 µM; n=8 for 127 µM; n=10 for 0, 7.5, and 40 µM.
IV. PEBBLEs

PEBBLEs produced using the precursors MTMS and PTMS, as described by Koo et al. (Koo et al., 2004) and in the Materials and Methods section were examined with a scanning electron microscope (Figure 37) and proved to be monodisperse and approximately 200 nm in diameter as expected. Various PEBBLE precursors beyond those used by Koo et al. (Koo et al., 2004) were tried and rejected outright because they either did not form PEBBLEs, had intractable problems with clumping or aggregation when dried, or would not retain the dyes. These precursors included Bis[3-(trimethoxysilane)-propyl]amino, 3-[2-(2-aminoethylamino)ethylamino] propyltrimethoxysilane, 1-[3-(trimethoxysilane)-propyl]urea, trimethoxysilane, and propyltrimethoxysilane.

An initial test of PEBBLEs produced using 20 µl of Tris-[3-(trimethoxysilane)-propyl]isocyanurate in place of the PTMS (20 µl of the isocyanurate hydrolyzed for 1.5 hr, then 3 ml NH₄OH, 10 µl FMA, 10 µl sulforhodamine added, incubated for 2.5 hr, then 100 µl MTMS added, incubate 3 hr, then filtered) suspended in polyacrylamide had a control red/green area ratio of 1.25±0.02 and a sulfide-exposed ratio of 2.53±0.3 (p<0.001, n=30 each). Figure 38 shows the results of a more extensive test with similar PEBBLEs. There was a strong response to cysteine in these PEBBLEs and thus no further testing was conducted.

Figure 39 is the result of a trial of PEBBLEs produced as described in the materials and methods but with the following modifications: 100 µl PTMS, 500 µl FMA, and 200 µl MTMS. The PEBBLEs were suspended in glycerol/acrylamide and incubated
Figure 37. Scanning electron micrograph of PTMS/MTMS PEBBLEs showing monodisperse sizing of approximately 200 nm
Figure 38 Quenching of MTMS/isocyanurate PEBBLEs suspended in glycerol/acrylamide. PEBBLEs were exposed to 100 µM Na₂S, 1 µM Na₂S, 1 mM cysteine, or nothing, then washed and resuspended in a mixture of glycerin and acrylamide/bisacrylamide. Notice that the PEBBLEs are quenched by cysteine, which precludes their use in cells. Polymerization of the acrylamide was initiated with sulfite, and a drop of the mixture immediately mounted on a slide for examination with the confocal microscope. n=3 slides each, 10 fields per slide. p=0.006 for control vs. 1 µM (*), and <0.001 for control vs. cysteine (**) and control vs. 100 µM (**).
Figure 39 Quenching of MTMS/PTMS PEBBLEs suspended in glycerol/acrylamide. These PEBBLEs were made with 100 µl PTMS, 500 µl FMA, 10 µl sulforhodamine, 200 µl MTMS. The results indicate that the PEBBLEs are essentially quenched completely in only 10 nM sulfide. The Na₂S was not washed out before imaging. Although there is a relatively large change from control to sulfide-exposed, leaching of the FMA from the PEBBLEs was a problem and required that later PEBBLEs use a lower concentration of FMA. n=3 slides each, 10 fields per slide. p<0.001 for all sulfide levels vs. control, different sulfide concentrations were not different from each other.
The graph shows the r/g area ratio for control, H$_2$S -8, H$_2$S -7, and H$_2$S -4 conditions.
at various concentrations of sulfide. These PEBBLEs show a good response and are essentially quenched completely at the lowest level tested, 10 nM Na$_2$S. Leaching proved to be a problem at the level of FMA used in these PEBBLEs, however, so subsequent batches used lower amounts.

Figures 40, 41, and 42 show the results of three experiments in which PEBBLEs were produced using 10, 15, or 20 µl FMA, respectively, and exposed to 1 mM cysteine or 100 µM Na$_2$S, then washed and mounted in a polyacrylamide/glycerol mixture. In each case the area ratios are not different between control and cysteine, while the area ratios are increased in the sulfide-exposed PEBBLEs. PEBBLEs produced with 15 µl FMA had the smallest standard deviation and largest change upon exposure to sulfide.

PEBBLEs were successfully deposited into cells with the custom-made gene gun (Fig. 43). To test viability of cells after being shot, bovine PASMC were shot in the lab, rinsed with PBS, and transferred to the cell culture room. Cells were trypsinized with 0.25% trypsin/EDTA and replated on coverslips with SMC medium supplemented with penicillin, streptomycin, and amphotericin B, and left overnight. The presence of morphologically normal, adherent, PEBBLE-containing cells confirmed that the cells were viable after shooting. An initial proof of principle test consisting of PEBBLEs shot into paraformaldehyde-fixed cells (glutaraldehyde resulted in excessive autofluorescence) and subsequently exposed to sulfide had a control red/green intensity ratio of $1.9 \pm 0.6$ and a sulfide-exposed ratio of $2.86 \pm 1.5$ ($p<0.001$; n control = 96, n sulfide = 81). In this case the intensities were found by visually intensity-bracketing the PEBBLEs in the Zen software.
Figure 40. Quenching of MTMS/PTMS PEBBLEs made with 10 µl FMA, suspended in glycerol/acrylamide. PEBBLEs were exposed to 1mM cysteine, 100 µM Na$_2$S, or nothing, then washed and resuspended in a mixture of glycerin and acrylamide/bisacrylamide. Polymerization of the acrylamide was initiated with sulfite, and a drop of the mixture immediately mounted on a slide for examination with the confocal microscope. Quenching with cysteine was not different from control while quenching with sulfide was significantly different from control and cysteine. n=3 slides each, 10 fields per slide.
Figure 41. Quenching of MTMS/PTMS PEBBLEs made with 15 µl FMA, suspended in glycerol/acrylamide. PEBBLEs were exposed to 1mM cysteine, 100 µM Na₂S, or nothing, then washed and resuspended in a mixture of glycerin and acrylamide/bisacrylamide. Polymerization of the acrylamide was initiated with sulfite, and a drop of the mixture immediately mounted on a slide for examination with the confocal microscope. Quenching with cysteine was not different from control while quenching with sulfide was significantly different from control and cysteine n=3 slides each, 10 fields per slide.
Figure 42. Quenching of MTMS/PTMS PEBBLEs made with 20 µl FMA, suspended in glycerol/acrylamide. PEBBLEs were exposed to 1mM cysteine, 100 µM Na$_2$S, or nothing, then washed and resuspended in a mixture of glycerin and acrylamide/bisacrylamide. Polymerization of the acrylamide was initiated with sulfite, and a drop of the mixture immediately mounted on a slide for examination with the confocal microscope. Quenching with cysteine was not different from control while quenching with sulfide was significantly different from control and cysteine (p<0.01. n=3 slides each, 10 fields per slide.)
Figure 43. Laser scanning confocal micrograph of breast cancer cells loaded with MTMS/PTMS PEBBLEs shot from the gene gun. Nuclei (purple) were stained with To-Pro-3.
Smooth muscle cells had excessive autofluorescence that prevented adequate measurement of PEBBLE fluorescence, thus breast cancer cells, which had less autofluorescence, were tried. Figure 44 shows the results of an experiment in which breast cancer cells were fixed immediately after being shot with PEBBLEs and then either exposed to nothing or exposed to H$_2$S, as well as cells that were shot and incubated alive in normoxic or hypoxic medium for 3 hr. The sulforhodamine 101/FMA fluorescence ratio significantly increased upon exposure to H$_2$S, relative to control (0.44±0.1 vs. 0.32 ± 0.07, p = 0.01). The ratio for air (0.31 ± 0.18) was not significantly different from control (p=0.89) but was different from H$_2$S (p=0.01). The ratio for N$_2$ (0.36 ±0.11) fell between that of control and H$_2$S but was not significantly different from any of the other three treatments (p=0.28, 0.06, 0.23 versus control, H$_2$S, and air, respectively).
Figure 44. Attempt at sulfide detection with MTMS / PTMS PEBBLEs in breast cancer cells. Cells were grown on coverslips in culture dishes. After removal of culture medium, cells were shot with PEBBLEs and either immediately fixed and exposed to nothing (con) or 100 µM Na$_2$S for ~15 min (H$_2$S), or incubated for 3 hr in air/5% CO$_2$ or N$_2$/5% CO$_2$. After incubation cells were fixed as with con and H$_2$S and all 4 treatments examined with the confocal microscope. H$_2$S was significantly different from control and from air (*), but was not different from N$_2$. N$_2$ was not significantly different from any other treatments. n= 3 each treatment, 10 fields per slide.
DISCUSSION

I. Amperometric H₂S Sensor Performance

It is evident from Figure 7A that the in-house sensor outperforms the commercial version both in terms of sensitivity and stability. Also, the cost of replacing the membrane, which is necessary at least weekly if used heavily, is ~twenty cents, vs. $40.00 for the WPI sensor. The sensor design went through numerous versions during the course of the research presented in this thesis, with improvements focused primarily on ease of use and durability, as acceptable sensitivity and stability were achieved early-on. The cost of the latest version of sensor is $50-70, which is almost entirely due to the platinum anode and cathode. Earlier versions used smaller-diameter wire for the anode, which was melted to form a bead at the tip that was subsequently ground down to its midpoint, providing a larger anode cross section while using the thinner (cheaper) wire. The thin wire required that a glass capillary tube be used for structural support, however, and it was fairly easy to break (an important consideration when undergraduates are to use the sensor). The thicker wire of the final version significantly increased the cost, but because it served as the structural support it eliminated the need for the fragile glass and made the sensor much more durable. Also, the wire is reusable if the glass sealing the anode happens to crack (the cracked glass can be removed by repeatedly heating in a flame and quenching in cold water). Multiple requests have been received from other labs interested in purchasing this sensor. Interferences determined so far include acetone.
(large spike that returns to baseline in a few minutes), dithiothreitol (increases sensor baseline), antimycin A (at 10 µM causes moderate and continuous rise in baseline that does not resolve with washes; membrane must be replaced), and cyanide (responds to CN⁻ similarly to H₂S but with ~10x lower sensitivity)

II. Blood and Plasma Measurement

II.A. Amperometric Measurement

There was no evidence of free H₂S in any blood tested (Table 2). In addition to failing to find appreciable concentrations of free sulfide in blood, it was also found that exogenous sulfide was rapidly removed by whole blood and, to a lesser extent, by plasma and BSA (Table 3). Surprisingly, however, when sulfide was added to whole blood the peak concentration measured typically did not exceed 20% of that which was added. As it was found that red blood cell ghosts did not affect sulfide disappearance, the rapid fall in sulfide likely reflects entry into the red blood cell where it may be bound to hemoglobin or metabolized. The slower exponential decrease in sulfide may indicate sulfide oxidation, as it is considerably faster in red blood cells containing mitochondria (lamprey and trout), and oxidation of sulfide by piscine mitochondria has been demonstrated (Bagarinao, 1990). These differences are not due to temperature effects on the amperometric H₂S sensor, as the sensor recovery after rapid sequestration of sulfide by addition of zinc acetate was faster at 37°C than at 15°C.
II.B. Colorimetric Measurement

A second methodology for sulfide measurement, the diffusion chamber colorimetric methylene blue method, was used in an attempt to replicate the results of many other papers that use this method (some earlier experiments in our lab were done with the direct method and gave similar results). Plasma sulfide levels were consistently less than 5 µM in all vertebrates (Table 4), values generally 10-fold lower than those reported elsewhere using similar methodologies (Appendix I). This included blood that was left 24 hrs before it was assayed, which was done to investigate whether the time delay would allow H$_2$S to accumulate from blood breakdown (not shown). To verify our methodology, blood was spiked prior to separation of the plasma. Sulfide was no higher in these samples than controls (i.e. recovery was zero), indicating that it had been consumed in the time it took to separate the plasma and further calling into question the likelihood of sulfide accumulation in the blood.

In a verification of this technique as performed here, plasma was spiked to 10 µM immediately prior to assay. Recoveries in this case were between 60 and 90% when the measured baseline sulfide was assumed to be additive to the spike. Recoveries this low were not expected, as spikes in Kreb’s buffer with 5% BSA showed complete recoveries (data not shown). If, however, the baseline sulfide concentration is assumed to be zero, as the flow-through data suggests, the average recovery in spiked plasma from all 30 samples is 101 ±3%. Thus it appears that there is no contribution from the measured baseline sulfide, i.e., 2 µM baseline + 10 µM Spike = 10 µM, not 12 µM. In agreement with this, a Spearman rank order correlation test found no relationship between spiked
values and their respective baselines, which theoretically should have been additive and strongly correlated (correlation coefficient = -0.07, p = 0.7). The control values are near the lower limit of detection for this method, and it is suspected that at these low levels sulfide is overestimated. Unfortunately it was not possible to replicate the results of the numerous studies that report such high sulfide levels using the methylene blue method, and it remains unknown how such studies arrive at those values. A point of concern with this method, although not an explanation of why the results here differed from the bulk of the literature, is that it measures total acid labile sulfide, which aside from H$_2$S, HS$^-$, and S$^{2-}$, also includes inorganic sulfide in iron-sulfur clusters of protein (Brumby et al., 1965) and possibly elsewhere (Ubuka 2002).

II.C. Sulfide-Ion Selective Electrode Measurement

The other commonly employed method of plasma sulfide measurement is the sulfide ion-selective electrode. The ISE requires a strongly alkaline environment to convert all H$_2$S and HS$^-$ to S$^{2-}$. This environment is obtained by using a sulfide antioxidant buffer (SAOB), originally described by Khan et al. (Khan, 1980) and the recipe for which is typically included in the purchase of the ISE. Khan’s report is frequently cited in papers that use this method for plasma measurement, but the author specifically states that directly mixing blood and the SAOB buffer greatly overestimates free sulfide, as the alkalinity of the buffer results in desulfuration of proteins via alkaline hydrolysis. When the authors assayed cow whole blood after directly mixing it with the antioxidant buffer, the measured sulfide was 100 µM and increased over time as the
desulfuration progressed. When an indirect method was employed, in which blood was acidified with HCl and the liberated H₂S carried to the antioxidant buffer in a stream of N₂, sulfide was only 2 µM. The research presented in this thesis did not attempt to use the ISE to measure baseline sulfide in plasma, but it did show unequivocally that, in agreement with Khan ((Khan, 1980), it should not be used for this purpose. Trout, lamprey, bovine, and ovine plasma as well as BSA were all shown to generate large quantities of sulfide in the SAOB (Fig 13-16). Transferring the ISE into a blank and then back into the plasma/SOAB solution shows clearly that the ISE signal is in fact due to sulfide production and not sensor drift. The presence of sulfide in the samples was also verified by the methylene blue method at the end of some ISE experiments. Further evidence comes from the obvious smell of H₂S gas after the samples were neutralized with HCl. The apparent consumption of sulfide following a 10µM spike (Fig. 17) can be explained if the exogenous sulfide is bound to sites on the protein other than those from which the sulfide is released by the SAOB. Because this method so grossly overestimates the sulfide concentration of plasma, any study which uses a different method (e.g. direct or indirect methylene blue) but reports similar values should be regarded with a high level of suspicion. The evidence above provides a convincing case against the presence of physiologically relevant free H₂S in blood. Following is a literature-based discussion supporting this contention.
II.D. Literature Support for Low Blood Levels

As can be seen in Appendix I, studies published prior to 2000 typically report that plasma (or blood) H$_2$S is 2 $\mu$M or less, whereas studies published after this date, with one exception, report plasma H$_2$S between 10 and 300 $\mu$M. The exception is an anecdotal report of $\leq$5 $\mu$M in rat blood measured with an amperometric H$_2$S sensor similar to ours (Koenitzer et al., 2007). The majority of the studies reporting undetectable or very low sulfide levels were published specifically to describe a technique for measuring sulfide in blood or plasma, or were published by authors who had previously described such a method. This adds some confidence to their conclusions because the methodologies presented were likely reviewed by fellow analytical chemists and thus held to a higher standard than papers citing previously developed methods. Studies that report higher levels of sulfide typically cite the direct (Siegel, 1965) or indirect (Stipanuk & Beck, 1982) methylene blue methods, which were not described for use in blood or plasma and have not been subsequently verified for such use. Alternately, they use the ISE and cite Khan et al. (Khan, 1980), who specifically said the method shouldn’t be used for blood, or cite manufacturer’s instructions, which describe the procedure of Khan et al. Thus the higher values reported post-2000 are not as credible as earlier, low values.

A second point is that 37 of the 47 studies I have found reporting sulfide levels above 5 $\mu$M used NaHS as a standard (one used (NH$_4$)$_2$S, two used Na$_2$S, four used H$_2$S, 3 unknown), while 12 of the 14 studies reporting less than 5 $\mu$M used Na$_2$S as a standard (one used H$_2$S, one used NaHS). NaHS cannot be obtained from common chemical suppliers in reagent grade quality, and has a variable hydration number while the
molecular weight is listed for the anhydrous form. It also tends to be contaminated (up to 50%) by sulfur species other than sulfide (Doeller et al., 2005), which is evident from the yellow discoloration of >1mM solutions. While titration of NaHS stock solutions to obtain an accurate sulfide concentration is straightforward, and a certificate of analysis can be obtained from suppliers, studies do not indicate that this has been done, and in any case that would not eliminate the contamination. Thus there is the potential for a standard curve that greatly overestimates the sulfide content of samples if the above factors are not taken into consideration. Na$_2$S, on the other hand, is available in 99.9% pure form (0.1M solutions are colorless), and has a constant hydration number.

An additional argument against the presence of significant quantities of sulfide in blood is that studies of H$_2$S toxicity reveal a robust capacity for detoxification. In pioneering studies, Haggard (Haggard, 1921) showed that quickly injecting 10 ml of 77 mM Na$_2$S into a dog was rapidly lethal whereas injecting five times that dose over 20 min left a dog “apparently none the worse”, leaving Haggard to conclude that sulfide was rapidly metabolized. Prior et al. (Prior et al., 1988) found in rats an inhaled LC$_{50}$ (concentration at which 50% of the subjects died) of 335 ppm over 6 hr while the LC$_{10}$ over the same time was 299 ppm. Assuming that H$_2$S readily equilibrates across the alveolar membranes, this would produce sulfide values in the plasma of 157 µM and 143 µM, respectively, according to Henry’s Law (see below; DeBruyn 1995). The authors suggest that the steepness of the dose-response curve is due to an overload of the H$_2$S detoxification system, implying that the majority of the lower dose is being continuously detoxified.
Given this capacity to metabolize sulfide, it is difficult to imagine that there would be any opportunity for sulfide to accumulate under resting conditions. It is also clear that H$_2$S can leave the blood across the alveolar membrane. For example, Evans injected anesthetized cats with a solution of NaHS and then used a CdSO$_4$ solution to trap the resulting exhaled H$_2$S, and subsequently assayed the trapped sulfide by iodometric titration (Evans, 1967). He found that at NaHS doses of 50-320 µmol/kg at rates of 9.3-88 µmol/kg/min he could recover ~5-25% of injected sulfide (with the exception of the slowest and smallest dose) when it was injected into a vein (external jugular, splenic, or femoral). Arterial injections had much lower recoveries.

With the knowledge that H$_2$S can enter the alveolar air from the blood, it is possible to predict exhaled H$_2$S given some concentration in the blood. Henry's Law states that at equilibrium the concentration of a gas dissolved in a liquid is proportional to the partial pressure of that gas in the headspace above the liquid (Boron & Boulpaep, 2005). The proportionality constant that relates these is termed the Henry's Law constant, and is a value specific to a given gas under a given set of conditions. Henry's Law constant for H$_2$S at 37°C and 140mM NaCl is 0.0649 M·atm$^{-1}$ (DeBruyn, 1995).

Assuming a blood H$_2$S concentration of 1.4µM (10 µM total sulfide at pH 7.4, 140 mM NaCl, 37°C, pK$_a$ 6.6) alveolar H$_2$S should be 2.2x10$^{-5}$ atm, or 22ppm (V/V) at sea level. Using similar Henry's Law data for CO$_2$ (Harned & Davis, 1943), the calculated partial pressure in alveolar air at 1.35 mM dissolved CO$_2$ in blood (normal level in humans) is 0.053 atm, or 5.3% at sea level. This is a typical value for alveolar CO$_2$ in humans, and supports the use of Henry’s Law to predict alveolar H$_2$S levels. Reported levels of H$_2$S
in human exhaled (Morselli-Labate et al., 2007) and end-expiratory air (Suarez et al., 1999; Furne et al., 2008) are ≤50 ppb, more than 400-fold less than predicted. In fact, 22 ppm is within the range of H₂S concentrations in the typical human flatus (Levitt et al., 1998) and slightly higher than the Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit (www.OSHA.gov 2007).

II.E. Conclusions About Sulfide in Blood

The evidence, both from the literature and from the data presented in this thesis, shows without ambiguity that free H₂S does not exist in appreciable quantities in blood. The amperometric H₂S sensor’s ~100 nM limit of detection at pH 7.4, as with the limit for most other methods, is far below the concentrations required to elicit physiologic responses in most studies. This leads to two conclusions: 1. H₂S does not act as a gasotransmitter in the blood and 2. Any distant effect of H₂S must actually be through some tightly-bound as yet unidentified species or a product of H₂S metabolism. With that said, there is also no question that a very large number of papers have found changes in the measured plasma “H₂S”, based on changes in pulmonary status (Chen et al., 2009), hypoxia (Perry et al., 2009), inflammation (Li et al., 2008), shock (Hui et al., 2003) hyperthermia (Han et al., 2005), myocardial infarction (Zhu et al., 2007), and various genetic and pharmacologic interventions (Appendix I). It is clear from the above discussion that free H₂S is not being measured in all these studies, but what is being measured is unknown. Evaluation of this awaits studies with S³⁵-labeled sulfide.
III. Tissues and Mitochondria

Because blood was found to be unsuitable for following H₂S changes with the amperometric H₂S sensor, it was necessary to look directly at tissues. This research found production in trout gill and heart, bovine heart and lung, and rat and seal lung. Reports in the literature find production from every organ system in numerous animals (Li and Moore, 2008; Olson, 2009; Szabó, 2007), but none report realtime changes in H₂S with respect to oxygen. The data presented here do, and provide the first direct measurements showing that there is an inverse relationship between O₂ and H₂S. This bolsters the previous arguments made by our lab that H₂S could act as an oxygen sensor, both in the pulmonary and systemic systems (Olson et al., 2006b; Olson et al., 2008a; Olson et al., 2008b)

III.A. Tissue Sulfide Consumption and Glutathione

The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is a marker of cell redox status, such that the ratio increases when oxygen decreases (Oktyabrsky & Smirnova, 2007). Artificially manipulating this ratio by fixing it in permeabilized cells or homogenates allows experimental control of redox status (Aon et al., 2007). Data presented here (Figures 20 and 21) indicate that the glutathione redox status can affect H₂S consumption in the absence of oxygen. In bovine lung homogenate which had been degassed with N₂, H₂S consumption was slowed relative to normoxic conditions. When GSSG was added to a concentration of 1 mM in anoxic conditions, the consumption rate greatly increased. If GSH was added, however, consumption ceased.
and the H$_2$S level either increased or was maintained at a relatively steady concentration. It was not possible to determine whether the source of the increasing H$_2$S was endogenous substrate, added glutathione, or added sulfide. The independence of sulfide consumption from oxygen levels demonstrates that mechanisms of sulfide metabolism exist outside the mitochondrial pathway on which most of the work here focuses.

A second experiment using reduced glutathione demonstrates the ease with which H$_2$S levels might be controlled by oxygen. When GSH was given to anoxic bovine lung tissue there was not any H$_2$S production observed, but when 10 µM Na$_2$S was added there was some production noted on top of the added sulfide. During this experiment the homogenate was maintained in anoxia by the presence of an N$_2$ source in the injection port of the chamber’s stopper. It was incidentally discovered that maintaining the stopper in a position so that the homogenate just touched the bottom of the injection port allowed titration of oxygen delivery to the homogenate by simply adding or removing the nitrogen source from the port. The physiologic relevance of this system requires that sulfide be endogenously produced, and while clearly showing the inverse relationship between oxygen and sulfide, it lacks endogenous production.

III.B. Tissue Consumption of Endogenous Sulfide and its Oxygen Dependence

The results here found cysteine-based production of hydrogen sulfide in numerous tissue homogenates. Steelhead (Fig 18) and bovine (Fig 19) heart were capable of H$_2$S production from 1 mM cysteine and 100 µM PLP, but only under near-anoxic conditions. The addition of air caused production to immediately reverse and become net
consumption, and upon depletion of oxygen, sulfide production resumed. Examination of
the traces for these and the lung homogenate experiments (Fig 22-23, 26, 27) shows just
how closely tied H$_2$S and oxygen are. In the case of bovine, seal, and rat lungs, PLP and
cysteine were not sufficient to cause production.

A recent paper has discussed the enzyme 3-mercaptopyruvate sulfurtransferase
(3-MST) in the context of H$_2$S production in the brain (Shibuya et al., 2009). 3-MST
cleaves the sulfhydryl group from 3-mercaptopyruvate, which in turn can be produced
from cysteine by transfer of its amino group to $\alpha$-ketoglutarate by cysteine
aminotransferase. It was reasoned that this could work in lung as well, and thus addition
of $\alpha$-ketoglutarate was tested as a means of stimulating H$_2$S production from cysteine. In
lung tissue from all three mammals tested, $\alpha$-ketoglutarate caused immediate production
of H$_2$S if cysteine was already present. If cysteine and $\alpha$-ketoglutarate were added in the
reverse order, production was not observed when $\alpha$-ketoglutarate was added alone, but
was observed once cysteine had also been added. In this case, however, there was
consistently a 2-4 minute delay between the addition of cysteine and initiation of
production. This delay has not been explained. Addition of a bubble of air immediately
caused H$_2$S consumption as above. It should be noted that the large standard errors of the
consumption rates, relative to production, are presumed to be related to the inconsistency
of the size of the injected bubble(s) of air. There was not an identifiable way to ensure
that each injection of air produced the same pattern of bubbles in the homogenate, thus in
some cases bubbles were larger and fewer while in other cases the converse was true.
This dramatically changes the surface area available for oxygen to diffuse into the bulk
solution. This was also evident in the large variability of the of the oxygen sensor response. The less than instantaneous kinetics of the O₂ and H₂S sensor responses do not allow precise measurement of the relationship between the two under these conditions, however it is obvious from inspection of the traces that H₂S consumption predominates unless the O₂ level is extremely low, certainly below 1 µM.

In order to further test the likelihood of 3-MST being involved, the same experiment was conducted with 1 mM succinate in place of α-ketoglutarate. This experiment had nearly identical results. Succinate is the second step past α-ketoglutarate in the citric acid cycle, and cannot act as an amine receptor. Succinate would have to run through the cycle back to α-ketoglutarate, which would require NAD⁺ molecules. In the anoxic environment of the experiment all the NAD molecules are likely to be in the reduced NADH form and so succinate traveling all the way through the cycle is unlikely to occur. The fact the succinate had the same effect as α-ketoglutarate argues against the hypothesis that 3-MST accounts for the H₂S production.

III.C. Lung Tissue Consumption of Exogenous Sulfide and its Oxygen Dependence

A recent review of potential oxygen sensors summarized reported oxygen levels at various positions from air to the cytosol (Ward, 2008). It appears that tissue oxygen levels range from 40-20 mmHg (~50-25 µM) while cytosolic oxygen levels were 25-10 mmHg (~30-13 µM). The level at mitochondria, where a significant portion of H₂S consumption occurs (Hildebrandt & Grieshaber, 2008), may be lower (see below). The endogenous production experiments discussed above make it appear that H₂S
consumption may in fact be inhibited during hypoxia, but it could not be accurately measured due to the presumed continued production of H₂S in the presence of oxygen as well as the inconsistent size of the bubbles and kinetic issues mentioned above.

These problems necessitated the use of exogenous sulfide and a preset oxygen level for determining the precise relationship between oxygen and H₂S. For these experiments a quantity of lung homogenate was added to homogenization buffer at the highest level that would allow for efficient bubbling without foaming. Homogenates in the chamber were bubbled with various mixtures of N₂ and air and then sealed, with no headspace. This was followed by injection of 2µM Na₂S. The oxygen consumption rate gradually fell from 40 µM O₂ (the highest [O₂] tested) to zero, decreasing more quickly after 10 µM. The H₂S consumption rate was lower than that of oxygen, and was steady from 40 µM O₂ to 20 µM, falling thereafter. The rate was half-maximum (P50) at ~4 µM O₂ (~3.2 mmHg). The higher rate of oxygen consumption vs. H₂S consumption most likely is due to the presence of other substrates and pathways. This also explains the inhibition of oxygen consumption at higher oxygen concentrations than those which inhibit H₂S consumption. See the discussion of the PASMC results and conclusions for discussion of the physiologic relevance of the above.

III.D. Mitochondrial Sulfide Consumption

As with lung homogenate, trout and bovine mitochondria consumed sulfide oxygen-dependently (Figs 29, 33). These experiments used the sulfidostat to maintain constant sulfide while evaluating both sulfide and oxygen consumption. Maintaining a
steady sulfide level over a few minutes was useful to determine more accurately the oxygen consumption rate and also to evaluate toxicity during the longer exposure to H₂S. That mitochondria oxidize sulfide has been known for decades (Beauchamp et al., 1984), although the pathway through which this took place was not elucidated until 2008 (Hildebrandt & Grieshaber, 2008). The pathway is described in the literature review. The research here found that trout liver mitochondria metabolized H₂S in conjunction with oxygen, with an O₂:H₂S ratio of 1.4 that showed no positive or negative trend from 0.5 to 20 µM (Fig. 30). The expected ratio is 1, with the difference here likely accounted for by residual substrate in the mitochondrial preparation. It has been shown that the O₂:H₂S ratio can vary at different oxygen levels, and thus this discrepancy is not concerning (Yong & Searcy, 2001). It is also unlikely that the type of mitochondria (trout liver) is an explanation for the discrepancy because Hildebrandt and Grieshaber identified the same series of steps in rat liver and Arenicola marina (lugworm) body wall mitochondria.

While the stoichiometry of H₂S consumption did not change with H₂S concentration, the rate of consumption by trout heart mitochondria did change. The maximum rate of consumption occurred at the lowest H₂S concentration, 0.5 µM, and the rate progressively decreased as the sulfide concentration increased (Fig. 31). This suggests that H₂S inhibits its own consumption, which is not surprising given that H₂S is known to inhibit cytochrome c oxidase (Beauchamp et al., 1984). It also brings up the possibility of a positive feedback situation in which low oxygen causes a small decrease in H₂S consumption rate, which allows slightly increased inhibition of its consumption,
etc. A similar concept has been postulated for nitric oxide, in which NO inhibition of complex IV increases the PO₂ at which mitochondria are effectively hypoxic (Hoffman et al., 2007). When the consumption rates were measured after 4 minutes of exposure to sulfide, the rate had decreased at all concentrations, indicating perhaps a combination of exhaustion of the mitochondria and progressive poisoning of cytochrome c oxidase. Trout heart mitochondria had very similar consumption rates to liver and suggests the generalizability of mitochondrial experiments.

In the case of bovine heart mitochondria the average O₂:H₂S ratio was 1.1 (Fig. 32), or ~1 if the ratios at 0.5 and 1 µM H₂S are excluded (they are statistically larger than the rest, discussed below). These mitochondria also did not show a trend in the stoichiometry with changing H₂S concentration, as expected. They metabolized sulfide much more quickly than trout mitochondria, partially due to the increased temperature of these experiments (37°C vs. 14°C). Calculated Q₁₀’s ranged from 2.5 to 3.5, while reported Q₁₀’s for mitochondrial oxidative phosphorylation are below 2 (Mootha et al., 1997). It seems, then, that temperature accounts for only a portion of the increased rate in bovine mitochondria. Bovine mitochondria showed the opposite trend shown by trout liver mitochondria with respect to H₂S consumption rate vs. H₂S concentration (Fig. 33). The rate steadily increased from 0.5 µM to 7µM, after which it was constant through 20 µM. The lack of inhibition at these levels is in line with the higher rates relative to trout mitochondria, whose mitochondria were inhibited at tested levels. After 4 minutes the pattern was similar up to 7 µM, but beyond that the rates began falling. The turning point at 7 µM presumably represents the level at which the increased rate from higher substrate
availability balances with mitochondrial inhibition (Goubern et al., 2007). Because of the relatively high level at which inhibition was seen, it is less likely for a positive feedback situation to develop because endogenous levels are probably much lower than 7 µM. On the other hand, in rat lung mitochondria it was found that at sulfide concentrations of 0.5, 1.0, and 2.0 µM cytochrome c oxidase showed 75%, 40%, and 22% of its control oxygen consumption (Khan et al., 1990). With respect to the higher O₂:H₂S ratio seen at 0.5 and 1 µM sulfide, a possible explanation is that a secondary pathway for oxygen consumption becomes maximally inhibited at 1 µM.

When bovine heart mitochondria H₂S consumption was examined as a function of oxygen concentration, there was little to no inhibition until the oxygen level reached zero (Fig. 34). The lowest non-zero oxygen level was 1.9 µM (~1.5 mmHg), which is near the limit of our equipment’s capabilities. There was no difference between the consumption rate at this level and 40µM O₂ (p=0.4). The rate of oxygen consumption, however, decreased from ~10 µM to zero, indicating that there was non-H₂S dependent O₂ consumption. This is reflected in an O₂:H₂S ratio much greater than 1 and is probably due to some residual substrates in the mitochondrial preparations, as mentioned earlier. If higher levels of alternate substrates were present (succinate, NADH), mimicking in vivo conditions, there would be more competition from reduced ubiquinone derived from complexes I and II, which would compete with the sulfide-quinone oxidoreductase, potentially increasing the oxygen level at which H₂S consumption began to slow. This would explain the higher P₅₀ for pulmonary tissue homogenate and PASMC.
Inhibition of H$_2$S consumption at such a low oxygen level may or may not be physiologic. Mik et al. used quenching of delayed fluorescence lifetime of mitochondrial protoporphyrin IX to determine in vivo cardiac mitochondrial oxygen tension (Mik et al., 2009). At 40% FiO$_2$ (required to achieve a normoxic PaO$_2$ of 115 mmHg), 10% of the mitochondria were at 0-10 mmHg, and 30% were at 11-20 mmHg, with an average of 35 mmHg. In hypoxia (10% FiO$_2$, equivalent to an altitude of ~5000 m), PaO$_2$ was 39 mmHg, 45% of mitochondria were at an oxygen tension of 0-10 mmHg (0-13 µM) and 10% were at 11-20 mmHg, with an average of 22 mmHg. At these levels it seems unlikely that H$_2$S consumption would be strongly inhibited. On the other hand, in vivo measurements of individual cardiomyocyte oxygenation in dog, cat, rabbit, ferret, and rat hearts, based on oxygen-dependent myoglobin absorbance in flash-frozen heart sections, found an average intracellular PO$_2$ of 3-10 mmHg, or 4-13 µM (Gayeski & Honig, 1991). The authors did not specify the ventilatory conditions, or even if the animals were ventilated. If it is assumed that the animals were normoxic, it would be expected that hypoxia would decrease tissue oxygen sufficiently to inhibit H$_2$S consumption.

In order to test the model that electrons from H$_2$S oxidation enter the electron transport chain at complex III, inhibitors of various complexes were tested in bovine heart mitochondria. Rotenone and 3-nitropropionic acid (inhibitors of complex I and II, respectively) did not significantly affect sulfide consumption, while inhibitors of complex III (antimycin A) and IV (sodium azide) greatly reduced sulfide consumption. This supports Hildebrandt and Grieshaber’s notion that a sulfide-quinone oxidoreductase reduces ubiquinone using electrons from sulfide, thus running parallel to NADH oxidase.
and succinate dehydrogenase (Hildebrandt & Grieshaber, 2008). It is also in line with reports that human colonocyte (Goubern et al., 2007) and chicken liver (Yong & Searcy, 2001) mitochondria can use sulfide to produce ATP.

III.E. Bovine Pulmonary Artery Smooth Muscle Cell Production and Consumption.

Tissue homogenates and mitochondrial preparations are clearly not ideal systems for measurement. A better system is cell culture. When whole bovine pulmonary artery smooth muscle cells were used (Fig 36), it was found that at arterial and venous PO2’s there was no difference in the rate of consumption of a 2 µM Na2S spike, while consumption decreased to 33% of control at 7.5 µM, 8% of control at 1.9 µM, and 3% of control at 0 µM O2. The P50 appears to be near 20 µM (~15 mmHg). The same percentages for lung homogenate were 75%, 33%, and 9% of control. Thus, in whole cells H2S consumption is inhibited at higher oxygen tensions than in homogenate (and isolated mitochondria). This can be explained by considering that in a homogenate there is stirring down to the level of mitochondria and enzymes, which eliminates the role of diffusion in transporting substrates to the mitochondria, while in the cells there is still a diffusion barrier from the membrane through the cytoplasm. Jones and Kennedy suggested such an effect while examining oxygen-dependent cytochrome c oxidase activity in free enzyme vs. whole cells (Jones & Kennedy, 1982).

I have been unable to find reports published that relate to the oxygen tension within pulmonary vascular tissue, but the average tissue PO2 must be only slightly lower than alveolar, as this is the case with blood. In intact rat lungs, hypoxic pulmonary
vasoconstriction (HPV), as determined by measurement of increasing pulmonary vascular resistance, begins to appear at alveolar PO$_2$s of ~60 mmHg (~80 µM), with a P$_{50}$ of 30 mmHg and a maximum at 10 mmHg (Marshall & Marshall, 1983). The authors showed that this effect was arterial, as hypoxia had no effect on perfusion pressure when the lungs were perfused from the pulmonary venous side, and that changes in alveolar PO$_2$ had a greater effect than changes in systemic venous PO$_2$ (Marshall & Marshall, 1983). This effect was explained by the greater area for diffusion of the outer portion of the vessel walls (larger diameter thus more surface area). Liu et al. found similar results in isolated 600-200 µm diameter pulmonary resistance arteries (Liu et al., 2001).

Contraction began at ~60 mmHg, with a P$_{50}$ of 40 mmHg and maximum response at ~20 mmHg. In isolated resistance PASMC, contraction took place at at least 50 mmHg (Madden et al., 1992). It thus appears that the PO$_2$s at which hypoxic vasoconstriction begins are higher than the PO$_2$s at which I found H$_2$S consumption to be inhibited in PASMC.

Unfortunately I could not reproducibly observe production of H$_2$S from cysteine in PASMC, trying up to 1 mM cysteine at 100-500k cells ml$^{-1}$. Cysteine levels above this are not close to physiologic and thus demonstrating production at the higher levels would not be useful. The experiments were performed in complete cell culture medium and thus there should not have been a lack of any additional required substrate. Doeller et al. (Doeller et al., 2005) reported production of H$_2$S from rat aorta smooth muscle cells, in complete medium, using an amperometric H$_2$S sensor. Production was reported to be 75 pmol min$^{-1}$ mg protein$^{-1}$, or very approximately 7.5 pmol min$^{-1}$ 1000 cells$^{-1}$ (based on the
given cell concentration of $10^5$-$10^6$ cells ml$^{-1}$, which is similar to the concentration I used) upon addition of cysteine and PLP into the closed respirometer. This level of production cannot be considered physiologic, however, because a physiologic state is already provided by the complete medium and thus cells should not require additional cysteine and PLP to produce a ‘normal’ amount of H$_2$S. Another point is that the oxygen was reported to remain at 4 µM for the duration of the experiment, and the provided oxygen trace showed no variation with addition of any reagents, during production of H$_2$S, or during consumption of H$_2$S after inhibition of H$_2$S production by $\beta$-cyanoalanine. It is unclear how oxygen would not decrease in a closed system with living cells, although a miscalibration is possible such that a level of zero oxygen was actually recorded as 4 µM.

III.F. Conclusions about sulfide production and consumption.

The ability of H$_2$S to serve as an oxygen sensor requires that 1. sufficient production of H$_2$S occurs at physiological concentrations of substrate and 2. intracellular oxygen levels drop low enough during hypoxia to inhibit H$_2$S consumption. With regard to the first point, it is not clear that this is the case. Lee et al. (Lee et al., 2004) found that regardless of a high- or low-protein diet, rat livers maintain intracellular cysteine between 20 and 100 nmol g$^{-1}$. Likewise, rat plasma cysteine is maintained between ~50-150 µM regardless of diet (Stipanuk et al., 2006). In humans, plasma cysteine has been reported to average near 300 µM (Salemi et al., 2009). This is in contrast to the situation found in most experiments involving H$_2$S production in tissues (including my work, our lab’s work, and that in the literature), in which cysteine concentrations of 1 mM or higher are
required to see production. It is possible that \textit{in vivo} production of H$_2$S is high enough due to rapid replacement of cysteine while maintaining it at a low level, but if that were the case experiments with the H$_2$S sensor should still have seen some production at lower levels.

With regard to the second point, there is a wide range of reported values for tissue PO$_2$ in mammals (Wilson, 2008) and thus an answer is not straightforward. Dyson \textit{et al.} (Dyson \textit{et al.}, 2007) used a phosphorescent fiber optic probe to measure the PO$_2$ in various rat tissues. The probe had a surface area of 8 mm$^2$ (i.e. it incorporated capillaries into its measurements), and measured, at 21% FiO$_2$/10% FiO$_2$, 55 mmHg/18 mmHg in muscle, 60 mmHg/7 mmHg in bladder, 20 mmHg/9 mmHg in liver, and 20 mmHg/15 mmHg in kidney. The unavoidable incorporation of capillaries (due to the high surface area) raised the measurements slightly. PO$_2$ in rat cremaster muscle is 27 mmHg at 21% FiO$_2$ and falls to 9 mmHg at 7% FiO$_2$, in this case measured using phosphorescence quenching of an injected dye (Johnson \textit{et al.}, 2005). Using yet another method (Mb desaturation measured via nuclear magnetic resonance), Richardson \textit{et al.} found in very lightly exercising quadriceps muscle a PO$_2$ of 3.5 mmHg during normoxia, vs. 2.3 mmHg when FiO$_2$ was reduced to 12% (Richardson \textit{et al.}, 1995). As mentioned earlier, Mik \textit{et al.} reported \textit{in vivo} cardiac mitochondrial PO$_2$ of 35 mmHg in normoxia and 22 mmHg at 10% FiO$_2$, with a much higher proportion of mitochondria at PO$_2$ of 0-10 mmHg during hypoxia. In a review article Ward reported a normoxic range for cytosolic PO$_2$ of $\sim$10-25 mmHg (Ward, 2008).
The above oxygen levels are generally near the upper end of the range at which I found inhibition of H$_2$S consumption and thus do not exclude the possibility of its physiologic relevance, but also do not argue strongly in favor of it. The higher levels of oxygen that begin producing HPV in PASMC and whole lungs tend to argue against H$_2$S as the sensor, because I have not found inhibition of its consumption at levels that high (~50 mmHg/65 µM). Another factor to consider is the level at which sulfide must be added to a system to see effects. Typically in the literature Na$_2$S or NaHS is added to experiments at “physiologic” levels (10-100 µM, roughly), the definition of physiologic being based on the understanding that blood normally carries these levels of H$_2$S (see literature review for examples). Data presented here refute those levels in blood. Studies also typically report production of sulfide in the tissue of interest, implying that those tissues make the H$_2$S they normally use. Despite the evidence that tissues make their own sulfide, experimenters typically add “physiologic” levels of sulfide to see an effect, when, in certain preparations (e.g. cell culture, whole animals) the effect should already be present from endogenous production. In most instances inhibitors of H$_2$S synthesis are used as well, and show an effect opposite to that of sulfide. Unfortunately the inhibitors are not specific for the stated enzymes (in particular AOAA and HA), and CBS and CSE are not specific to H$_2$S production. Thus, effects attributed to decreased H$_2$S might be due to other metabolic derangements. For example, many studies use AOAA to inhibit the malate-aspartate shuttle (MAS) via mitochondrial transaminases, and while the intent of the authors obviously does not change what a given inhibitor does, experimental
effects are not interpreted as a combination of MAS inhibition and H₂S depletion despite the fact that both happen.

The weight of the evidence seems to point toward very low levels of intracellular H₂S, at least on average across a cell. An untested possibility is that localized areas of the cell, i.e. those farther away from mitochondria, have higher levels of H₂S that may be active. In conjunction, ROS-type signals as well as NO and CO tend to function at much lower levels than those typically reported for H₂S. If this is the case, then current modes of detection are insufficient for measurement under physiologic situations. That was the impetus for my attempt to develop PEBBLE sensors for H₂S, discussed below.

As should be obvious at this point, tissues seem to be strongly biased towards consumption of H₂S rather than its production.

IV. PEBBLEs

The most basic requirements for PEBBLEs to function are sensitivity to H₂S and insensitivity to other small thiols (e.g. cysteine). The PEBBLEs developed here meet those criteria (Figures 40-42). The sensing dye, fluorescein mercuric acetate, is permanently quenched by H₂S, which has both advantages and disadvantages. On one hand, it does not allow real-time monitoring of H₂S levels, but on the other hand it means that the quenching is cumulative and thus allows even very small amounts of sulfide to be measured, if given sufficient time. While not theoretically limiting with respect to accurate concentration measurement, practically speaking it limits their potential usefulness to identifying the presence or absence of sulfide. Based on the bulk of the
evidence presented in this thesis, normoxic conditions preclude the accumulation of any sulfide, while hypoxic conditions may allow its accumulation, thus the ability to simply identify the presence of sulfide could be useful.

Unfortunately, while meeting the basic requirements mentioned above, the PEBBLEs did not successfully detect sulfide in living cells (Figure 44). The slightly increased ratio in the N₂-treated cells is suggestive of the presence of sulfide, but the variability is too high to approach significance (p=0.28 relative to control). The major obstacle to these PEBBLEs functioning as planned was FMA’s weak quenching when inside the PEBBLEs. FMA free in solution is almost completely quenched by sulfide, while FMA incorporated into PEBBLEs typically lost only ~15% of its fluorescence when maximally quenched. This presumably is due to a combination of inaccessibility of H₂S to the FMA within the PEBBLE matrix as well as a non-optimal environment for the quenching reaction. Forming the PEBBLEs in the presence of a much higher concentration of FMA increased the quenching substantially (Figure 39 vs. Figures 40-42), but also resulted in a substantial amount of dye leaching, as evidenced by green fluorescence in the PEBBLE buffer.

The weak quenching creates a problem with respect to the signal-to-noise ratio. Even in the homogeneous environment of the glycerol/polyacrylamide mount, there was a high variability in the sulforhodamine/FMA ratio measurements relative to the change associated with complete quenching. An attempt was made to measure partial quenching of PEBBLEs (Figure 39) and while it appears that quenching was essentially complete at all sulfide levels, variability is high enough to preclude measuring differences between
sulfide levels. When measured in the heterogeneous intracellular environment, the variability in ratio measurements was also too high to obtain significant measurements unless the PEBBLEs were completely quenched. This is evident in the size of the error bars in Figure 44, which appear relatively large despite an n of 30 (3 slides with 10 fields per slide). The much lower red/green ratio in Figure 44 relative to Figures 40-42 reflects the predominance of green autofluorescence in the breast cancer cells. The relatively weak fluorescence of FMA when incorporated into the PEBBLEs also necessitated the use of higher laser intensities than desirable, which exacerbated autofluorescence.

Despite these problems, the difference between the control and fully quenched PEBBLEs in fixed cells was significant (both in Figure 44 and in the proof-of-principle experiment described in Results), indicating that if sufficient H$_2$S was produced in the living cells it should be possible to detect it. Perhaps the 3 hr incubation time allowed in this experiment was not long enough, although it is questionable how physiologically relevant longer periods of near-anoxia would be. A second possibility is that the cells were no longer viable, although this is unlikely because there were no obvious morphological changes and in prior viability testing cells could be successfully trypsinized and replated after loading with PEBBLEs. The culture medium has 100 µM cysteine in it, which is physiologic, but is lower than the ≥1 mM cysteine used in most H$_2$S production experiments. A higher level of cysteine may have resulted in a measurable sulfide production, but again this would not have been a physiologic situation.
As mentioned in the results section, numerous PEBBLE precursors were tried and rejected in the hope of creating PEBBLEs with a better environment for quenching than the MTMS / PTMS PEBBLEs. Tris-[3-(trimethoxysilane)-propyl] isocyanurate is the only precursor besides MTMS and PTMS which showed promise. PEBBLEs made using the isocyanurate precursor were quenched more completely than PTMS / MTMS PEBBLEs, but as shown in Figure 38, they were also quenched by cysteine. The sensitivity to cysteine is most likely due to a larger pore size created by the bulkier organic groups of this precursor. This unfortunately precluded their use in cells.

The goal of developing the PEBBLEs was to create a means to detect endogenous, physiologically produced sulfide and establish whether hydrogen sulfide does in fact increase during hypoxia. While this goal has not yet been achieved, the PEBBLEs described here do show that it is possible to biolistically deposit the PEBBLEs within a cell and use them to detect sulfide, albeit when the sulfide is exogenously applied. Further experimentation under different incubation conditions and/or different cell lines may prove fruitful. Additionally, refinement of the intensity measurement technique is needed in order to decrease the high variability during intracellular measurements. Koo et al. (Koo et al., 2004) use a spectrograph for this purpose, which allows them to measure the spectrum of the PEBBLEs, and take intensity measurements at the peak emission. This technique may help separate the PEBBLE signal from the background. Ultimately a reversibly-quenched dye is needed to allow real-time measurements of sulfide.
After completion of the research reported in this thesis, a paper was published that used a sol-gel process similar to that used in PEBBLE production to form sulfide-sensitive PbO/SiO₂ particles (Zhou et al., 2010). These particles phosphoresce green when exposed to UV light, and the phosphorescence is permanently quenched by sulfide. The authors compressed the particles into ~12 x 2 mm discs and examined the discs’ suitability for sulfide measurement, finding that they could quantitatively detect sulfide below 1 µM. They verified their sensor’s performance by measuring samples in parallel with the methylene blue method, arriving at very similar values. There was no indication of how the particles would function individually, as PEBBLEs are designed to work, but the PbO/SiO₂ method of sulfide detection is a promising avenue for future research.
SUMMARY

The work presented here clearly demonstrates the lack of circulating H₂S in mammals, trout, and lamprey, contrasting with the majority of recent studies but confirming many older studies (Appendix I). It is also evident that blood rapidly consumes sulfide, further undermining recent reports that show substantial H₂S levels in blood and again confirming older work. An explanation for some of the unexpectedly high reported sulfide levels is that the measurement method used, a sulfide ion-selective electrode in sulfide antioxidant buffer, produces sulfide from the cysteines of various plasma proteins. This was shown here in a few types of plasma and in bovine serum albumin. The absence of H₂S from the blood and its inability to accumulate there precludes free H₂S from serving as a circulating gasotransmitter and limits its potential actions to paracrine or autocrine.

Also shown in this thesis is the sensitivity of hydrogen sulfide to the presence of oxygen in biological systems. The H₂S sensor developed during the course of this research has allowed real-time demonstrations of the production of H₂S from cysteine in various tissues, generally under near-anoxic conditions, and the consumption of that H₂S in the presence of oxygen. This is consistent with the role of H₂S as a potential oxygen sensor. The sensitivity to oxygen is high enough, however, that sulfide consumption in excess of production occurs at oxygen levels very near or below the lower end of those
which would be expected to be found in living cells, even in the face of supraphysiologic levels of cysteine. These facts seem to preclude the functioning of H$_2$S at any more than the smallest scales within a cell.

An effort to address the intracellular production of H$_2$S was made through the development of sulfide-sensitive PEBBLEs. This technique was intended to allow localization of H$_2$S production within cells by measuring fluorescence quenching in the nano-scale PEBBLEs. The PEBBLEs were capable of detecting sulfide in acellular media and in killed cells provided with exogenous sulfide, but did not detect sulfide produced in living cells under low-oxygen conditions. The technique is not refined enough to conclude that the cells did not produce sulfide, however. There is much work left to be done in evaluating the potential of this technique, which includes better microscopy techniques, alterations in the protocol for PEBBLE production, and different experimental conditions and cell types. A related avenue of research should examine newly-reported nano-scale sulfide-sensitive particles that uses PbO/SiO$_2$ phosphorescence quenching for detection (Zhou et al., 2010).
APPENDIX I, STUDIES REPORTING PLASMA CONCENTRATIONS OF H₂S

Appendix I does not include cases of lethal H₂S poisoning or those in which the reductant dithiothreitol was added to the sample prior to analysis. ‘Sample’ column indicates the source of blood or plasma; ‘Assay Method (Source)’ column gives the assay method used followed by the source cited for that method; ‘Sample Site’ column indicates from which site the sample was obtained; ‘Standard’ column indicates the source of sulfide used for standards; ‘[H₂S]’ column gives the control plasma or blood concentration of H₂S in µM, unless otherwise noted; ‘Experimental Changes’ column indicates the changes in H₂S reported for various experimental manipulations; ‘DL’ column indicates the lower limit of detection for the method, if provided; Question mark indicates that information was not provided. Direct ISE, sample is added directly to sulfide antioxidant buffer; Indirect ISE, sample is acidified and H₂S carried to antioxidant buffer in a stream of N₂; direct methylene blue, sample is added directly to assay reagents; indirect methylene blue, H₂S is liberated from sample with acid and trapped as ZnS on filter paper which is then added to assay reagents.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay Method (Source)</th>
<th>Sample Site</th>
<th>Standard</th>
<th>[H₂S]</th>
<th>Experimental Changes</th>
<th>DL</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep blood</td>
<td>imb (Conway, 1957)</td>
<td>jugular vein</td>
<td>Na₂S</td>
<td>N/D</td>
<td>no change with giving sulfide or changing feed</td>
<td>?</td>
<td>(Spais et al., 1968)</td>
</tr>
<tr>
<td>Wistar rat blood</td>
<td>direct ISE (none)</td>
<td>?</td>
<td>Na₂S</td>
<td>10</td>
<td>giving high Mo decreased S oxidizing cap but didn’t change blood sulfide</td>
<td>?</td>
<td>(Mason et al., 1978)</td>
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<tr>
<td>steer blood</td>
<td>indirect ISE (d.m.)</td>
<td>jugular vein</td>
<td>Na₂S</td>
<td>2</td>
<td>N/A</td>
<td>?</td>
<td>(Khan, 1980)</td>
</tr>
<tr>
<td>Wistar rat blood</td>
<td>alk ext dx PFBBr, GC-ECD (d.m.)</td>
<td>ear vein</td>
<td>Na₂S</td>
<td>N/D</td>
<td>increased to 14 after death by 600 ppm H₂S made from Na₂S</td>
<td>0.3</td>
<td>(Nagata et al., 1990)</td>
</tr>
<tr>
<td>Wistar rat blood</td>
<td>alk ext dx PFBBr, GC-ECD (Kage 1988)</td>
<td>?</td>
<td>Na₂S</td>
<td>N/D</td>
<td>500-1000 ppm by inhalation (lethal) = 0.012 µmol/g, 200 ppm (non lethal)= N/D</td>
<td>0.3</td>
<td>(Kage, 1992)</td>
</tr>
<tr>
<td>human serum</td>
<td>dx MBB, HPLC (d.m.) ²</td>
<td>?</td>
<td>Na₂S</td>
<td>N/D</td>
<td>unknown</td>
<td>0.1</td>
<td>(Togawa et al., 1992)</td>
</tr>
<tr>
<td>rabbit blood</td>
<td>alk ext dx PFBBr, GC-ECD (Kage et al., 1988)</td>
<td>ear vein</td>
<td>Na₂S</td>
<td>N/D</td>
<td>7 and 30 in fatal poisoning</td>
<td>0.3</td>
<td>(Kage et al., 1997)</td>
</tr>
<tr>
<td>human plasma</td>
<td>dx DEAT, HPLC (d.m.) ²</td>
<td>?</td>
<td>Na₂S</td>
<td>0.03</td>
<td>N/A</td>
<td></td>
<td>(Nagashima et al., 1993)</td>
</tr>
<tr>
<td>human serum</td>
<td>microdist, NaOH trap, titrate with I₂ (d.m.)</td>
<td>?</td>
<td>Na₂S</td>
<td>N/D</td>
<td>added 5, 10, 15 µg / 50 ml, recovered all</td>
<td>0.13</td>
<td>(Puacz et al., 1995)</td>
</tr>
<tr>
<td>human blood</td>
<td>alk ext dx PFBBr, GC-ECD (Kage et al., 1988)</td>
<td>?</td>
<td>Na₂S</td>
<td>N/D</td>
<td>unknown</td>
<td></td>
<td>(Kage et al., 1997)</td>
</tr>
<tr>
<td>human serum</td>
<td>microdist, ion chrome, e.c.d. (d.m.)</td>
<td>?</td>
<td>(NH₄)₂S</td>
<td>2-6, 42, 47, 52e</td>
<td>no change when changing protein in diet</td>
<td>?</td>
<td>(Richardson et al., 2000)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>ISE (Khan, 1980)</td>
<td>femoral v or carotid a</td>
<td>H₂S or NaHS</td>
<td>45.6</td>
<td>N/A</td>
<td></td>
<td>(Zhao et al., 2001)</td>
</tr>
<tr>
<td>human blood</td>
<td>alk ext dx PFBBr, GC-MS (d.m.)</td>
<td>?</td>
<td>Na₂S</td>
<td>53.5</td>
<td>in vitro spiked baseline 53 by additional 12.5, 25, 37.5 and recovered most</td>
<td>?</td>
<td>(Hyspler et al., 2002)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (none)</td>
<td>jugular vein or carotid artery</td>
<td>NaHS</td>
<td>301</td>
<td>drop to 192 in hypoxia, up to 317 in hypoxia + NaHS</td>
<td>?</td>
<td>(Zhong et al., 2003)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>ISE or methylene blue? (Stipanuk &amp; Beck, 1982; Zhao et al., 2001)</td>
<td>jugular vein</td>
<td>NaHS</td>
<td>38.6</td>
<td>decreased ~50% with L-NAME treatment</td>
<td>?</td>
<td>(Zhong et al., 2003)</td>
</tr>
<tr>
<td>Sample</td>
<td>Assay Method (Source)</td>
<td>Sample Site</td>
<td>Standard</td>
<td>[H₂S]</td>
<td>Experimental Changes</td>
<td>DL</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------</td>
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<td>----------</td>
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<td>----</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>trout plasma</td>
<td>direct ISE (mfr. directions)</td>
<td>hemal arch</td>
<td>NaHS</td>
<td>38</td>
<td>N/A</td>
<td>?</td>
<td>(Dombkowski et al., 2004)</td>
</tr>
<tr>
<td>trout plasma</td>
<td>dmb (Siegel, 1965; Zhang et al., 2003)</td>
<td>hemal arch</td>
<td>NaHS</td>
<td>40</td>
<td>N/A</td>
<td>?</td>
<td>(Dombkowski et al., 2004)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (Stipanuk &amp; Beck, 1982)</td>
<td>left ventricle</td>
<td>NaHS</td>
<td>60</td>
<td>drop to ~20 with isoproterenol treatment</td>
<td>?</td>
<td>(Geng et al., 2004)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>dmb (Stipanuk &amp; Beck, 1982)</td>
<td>femoral artery</td>
<td>NaHS</td>
<td>28.9</td>
<td>increased to 37 after hemorrhage</td>
<td>?</td>
<td>(Mok et al., 2004)</td>
</tr>
<tr>
<td>SHR rat plasma</td>
<td>dmb (none)</td>
<td>carotid artery</td>
<td>NaHS</td>
<td>20</td>
<td>say that this is decrease vs the WKY control below (H₂S lower in hypertensive rats)</td>
<td>?</td>
<td>(Yan et al., 2004)</td>
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<tr>
<td>WKY rat plasma</td>
<td>dmb (none)</td>
<td>carotid artery</td>
<td>NaHS</td>
<td>48</td>
<td>see above</td>
<td>?</td>
<td>see above</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (Zhang et al., 2003)</td>
<td>?</td>
<td>NaHS</td>
<td>293</td>
<td>drops to 196 in hypoxia, up to 323 in hypoxia+NaHS, down to 124 in hypoxia+PPG</td>
<td>?</td>
<td>(Zhang et al., 2004; Chen et al., 2005)</td>
</tr>
<tr>
<td>human plasma</td>
<td>direct ISE (Zhao et al., 2001)</td>
<td>?</td>
<td>H₂S or NaHS</td>
<td>34</td>
<td>increased to in stable COPD patients</td>
<td>?</td>
<td>(Chen et al., 2005)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (none)</td>
<td>?</td>
<td>NaHS</td>
<td>290</td>
<td>increased to 369 in 45.2°C temperature-induced seizures, decreased to 126 with seizures+HA, increased to 456 with seizures and NaHS</td>
<td>?</td>
<td>(Han et al., 2005)</td>
</tr>
<tr>
<td>human plasma</td>
<td>direct ISE</td>
<td>-------</td>
<td>-------</td>
<td>51.7</td>
<td>decreased to 26 μM in heart disease pts</td>
<td>?</td>
<td>(Jiang et al., 2005)</td>
</tr>
<tr>
<td>mouse plasma</td>
<td>dmb (Mok et al., 2004)</td>
<td>cardiac puncture</td>
<td>NaHS</td>
<td>34</td>
<td>increased to 41 h after LPS, and 65 24 h after LPS</td>
<td>?</td>
<td>(Li et al., 2005)</td>
</tr>
<tr>
<td>human plasma</td>
<td>dmb (Mok et al., 2004)</td>
<td>?</td>
<td>NaHS</td>
<td>43.8</td>
<td>increased to 150 in humans with septic shock</td>
<td>?</td>
<td>see above</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>dmb (none)</td>
<td>jugular</td>
<td>NaHS</td>
<td>51</td>
<td>2 weeks of high pulm flow from aorto-caval shunt decreased plasma H₂S to 36</td>
<td>?</td>
<td>(Xiaohui et al., 2005)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>ion int rev ph HPLC (Savage &amp; Gould, 1990)</td>
<td>?</td>
<td>NaHS</td>
<td>18.9</td>
<td>increased to 22.7 h LPS, 24.1 LPS plus ascorbate</td>
<td>?</td>
<td>(Mitsuhashi et al., 2005)</td>
</tr>
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<td>SD rat plasma</td>
<td>dmb (Mok et al., 2004)</td>
<td>trunk blood</td>
<td>NaHS</td>
<td>40</td>
<td>decreased to 38 with streptozocin treatment and increased to 42 with stz +insulin</td>
<td>?</td>
<td>(Yusuf et al., 2005)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>indirect ISE (Khan, 1980; Zhao et al., 2001)</td>
<td>?</td>
<td>NaHS</td>
<td>47</td>
<td>decreases to ~37 with colorectal distension (i.e. pain), 29 with i.p. NaHS, 18 with NaHS + L-NAME</td>
<td>?</td>
<td>(Distrutti et al., 2006)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb none</td>
<td>?</td>
<td>NaHS</td>
<td>298</td>
<td>decreases to 195 with hypoxia, 271 with i.p. NaHS + hypoxia</td>
<td>?</td>
<td>(Jin et al., 2006)</td>
</tr>
<tr>
<td>human child plasma</td>
<td>direct ISE (none)</td>
<td>?</td>
<td>NaHS</td>
<td>65</td>
<td>decreased to 52 in children with essential HTN</td>
<td>?</td>
<td>(Chen et al., 2007)</td>
</tr>
<tr>
<td>Sample</td>
<td>Assay Method (Source)</td>
<td>Sample Site</td>
<td>Standard</td>
<td>[H$_2$S]</td>
<td>Experimental Changes</td>
<td>DL</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
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<td>--------------------------------------------------------------------------------------</td>
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<tr>
<td>C57BL/6 mice plasma</td>
<td>dmb (Yan et al., 2004)</td>
<td>?</td>
<td>NaHS</td>
<td>24</td>
<td>injection, increased over time to max 45 at 1 h, back to baseline at 6</td>
<td>?</td>
<td>(Cai et al., 2007)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>ISE (Zhao et al., 2001)</td>
<td></td>
<td>NaHS</td>
<td>46</td>
<td>N/A</td>
<td>?</td>
<td>(Geng et al., 2007)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (Lowicka &amp; Beltowski, 2007) (this ref doesn’t list a method)</td>
<td>R ventricle</td>
<td>NaHS</td>
<td>27.1</td>
<td>MI: saline 36, SAC 43.4, SAC/PPG 33.3, PPG 26.3. Sham: SAC 30, SAC/PPG 25.5, PPG 22.9</td>
<td>?</td>
<td>(Chuah et al., 2007)</td>
</tr>
<tr>
<td>human child plasma</td>
<td>direct ISE</td>
<td>----</td>
<td>- - -</td>
<td>52</td>
<td>N/A</td>
<td>?</td>
<td>(Ding et al., 2006)</td>
</tr>
<tr>
<td>SD rat blood</td>
<td>AHSS (Doeller et al., 2005)</td>
<td>?</td>
<td>Na$_2$S</td>
<td>&lt;5</td>
<td>increases to 20 with cecal ligation / puncture</td>
<td>?</td>
<td>(Koenitzer et al., 2007)</td>
</tr>
<tr>
<td>SA mouse plasma</td>
<td>dmb (none)</td>
<td>?</td>
<td>NaHS</td>
<td>8</td>
<td></td>
<td>?</td>
<td>(Zhang et al., 2007)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (Mok et al., 2004; Li et al., 2005)</td>
<td>?</td>
<td>NaHS</td>
<td>38</td>
<td>increased after MI as follows (all i.p.): vehicle 57, PPG 39, NaHS 92</td>
<td>?</td>
<td>(Zhu et al., 2007)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>ISE (Wedding, 1987)</td>
<td>abdominal aorta</td>
<td>H$_2$S</td>
<td>29</td>
<td>“HES solution” sub q increased to 40, homocysteine sub Q increased to 50</td>
<td>?</td>
<td>(Chang et al., 2008)</td>
</tr>
<tr>
<td>Sw. alb. mouse plasma</td>
<td>dmb (none)</td>
<td>?</td>
<td>NaHS</td>
<td>14</td>
<td>sham PPG 13, increased to 20µM in CLP, 8 in CLP+PPG</td>
<td>?</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>dmb (none)</td>
<td>?</td>
<td>NaHS</td>
<td>300</td>
<td>decreased to 187 in hypoxia</td>
<td>?</td>
<td>(Wei et al., 2008)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>dmb (Li et al., 2005)</td>
<td>femoral artery</td>
<td>NaHS</td>
<td>35, 45</td>
<td>78µM 30 min after GYY4137 admin, no change when 20µmol/kg NaHS IP</td>
<td>?</td>
<td>(Li et al., 2008a)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>ISE (Geng et al., 2007)</td>
<td>abdominal aorta</td>
<td>NaHS</td>
<td>37</td>
<td>ovalbumin 20, ovalbumin+NaHS 27</td>
<td>?</td>
<td>(Li et al., 2008c)</td>
</tr>
<tr>
<td>C57BL/6 mice plasma</td>
<td>ISE (Zhao et al., 2001; Geng et al., 2005; Chen et al., 2007)</td>
<td>apical cardiac puncture</td>
<td>Na$_2$S</td>
<td>23.8</td>
<td>80 ppm inhaled 30 minutes didn’t change baseline, 200 ppm increased to 24.2</td>
<td>?</td>
<td>(Volpato et al., 2008)</td>
</tr>
<tr>
<td>human</td>
<td>GC-CL (none)</td>
<td>alveolar air</td>
<td>Na$_2$S</td>
<td>1.33 ppb</td>
<td>N/A</td>
<td>1 ppb</td>
<td>(Furne et al., 2008)</td>
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<tr>
<td>C57BL/6J 129SvEv mice plasma</td>
<td>ISE (mfg inst)</td>
<td>?</td>
<td>NaHS</td>
<td>40</td>
<td>decrease to 34 in CSE +/-, 18 in +/-</td>
<td>?</td>
<td>(Yang et al., 2008)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>dmb with HPLC det.(Li et al., 2005a)</td>
<td>jugular vein or vena cava</td>
<td>NaHS</td>
<td>0.55</td>
<td>1.4 after administration of H$_2$S-releasing drug</td>
<td>&lt;0.2</td>
<td>(Sparatore et al., 2009)</td>
</tr>
<tr>
<td>rainbow trout plasma</td>
<td>ISE (mfg inst)</td>
<td>dorsal aorta</td>
<td>NaHS</td>
<td>90</td>
<td>420 in hypoxia</td>
<td>?</td>
<td>(Perry et al., 2009)</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>ISE (none)</td>
<td>NaHS</td>
<td>58</td>
<td></td>
<td>apoE +/- mice: 45, +NaHS 52, +PPG 36</td>
<td>?</td>
<td>(Wang et al., 2009)</td>
</tr>
<tr>
<td>Zucker diabetic fatty rat plasma</td>
<td>ISE (Khan, 1980)</td>
<td>?</td>
<td>H$_2$S</td>
<td>68</td>
<td>decreased to 19 with PPG</td>
<td>?</td>
<td>(Wu et al., 2009)</td>
</tr>
<tr>
<td>Sample</td>
<td>Assay Method (Source)</td>
<td>Sample Site</td>
<td>Standard</td>
<td>[H₂S]</td>
<td>Experimental Changes</td>
<td>DL</td>
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<tr>
<td>Zucker fatty rat plasma</td>
<td>ISE (Khan, 1980)</td>
<td></td>
<td>H₂S</td>
<td>49</td>
<td>decreased to 24 with PPG</td>
<td>?</td>
<td>see above</td>
</tr>
<tr>
<td>Zucker lean rat plasma</td>
<td>ISE (Khan, 1980)</td>
<td></td>
<td>H₂S</td>
<td>49</td>
<td>decreased to 20 with PPG</td>
<td>?</td>
<td>see above</td>
</tr>
<tr>
<td>SD-rats</td>
<td>ISE (Zhao et al., 2001)</td>
<td></td>
<td>NaHS</td>
<td>31</td>
<td>ovalbumin-treated up to 72, ova with NaHS to 40</td>
<td>?</td>
<td>(Chen et al., 2009)</td>
</tr>
<tr>
<td>C57BL/6J mouse plasma</td>
<td>dmb (Yang et al., 2007)</td>
<td></td>
<td>NaHS</td>
<td>56</td>
<td>no change 2-kidney wt+30µM in drinking water, 38 in CBS++, back to 56 when NaHS in water</td>
<td>?</td>
<td>(Sen et al., 2009)</td>
</tr>
<tr>
<td>New Zealand white rabbit plasma</td>
<td>dmb (Chuah et al., 2007)</td>
<td>ear vein</td>
<td>NaHS</td>
<td>16.5</td>
<td>N/A</td>
<td>?</td>
<td>(Srilatha et al., 2009)</td>
</tr>
<tr>
<td>SD rat plasma</td>
<td>dmb (Lowicka &amp; Beltowski, 2007)</td>
<td>abdominal aorta</td>
<td>?</td>
<td>43</td>
<td>SPRC+MI = 51.6, SAC+MI = 45.4, vehicle+MI = 39.9, SPRC+MI+PPG = 37.2, SAC+MI+PPG = 35.7, vehicle+MI+PPG = 29</td>
<td>?</td>
<td>(Wang et al., 2009)</td>
</tr>
<tr>
<td>human plasma</td>
<td>ISE (Zhao et al., 2001)</td>
<td></td>
<td>?</td>
<td>35</td>
<td>22.7 in pneumonia, 22.8 in COPD</td>
<td>?</td>
<td>(Chen et al., 2009)</td>
</tr>
<tr>
<td>Balb/C mouse plasma</td>
<td>dmb (Zhang et al., 2006)</td>
<td>NaHS</td>
<td>25</td>
<td></td>
<td>myocarditis from coxsakievirus B3 infection= 60, infected + PPG = 35, infected + NaHS = 75</td>
<td>?</td>
<td>(Hua et al., 2009)</td>
</tr>
<tr>
<td>human plasma</td>
<td>dmb (Chunyu et al., 2003)</td>
<td>venous</td>
<td>NaHS</td>
<td>14.5</td>
<td>chronic renal failure patients = 11</td>
<td>?</td>
<td>(Perna et al., 2009)</td>
</tr>
</tbody>
</table>

Abbreviations: AHSS, amperometric hydrogen sulfide sensor; alk ext, alkaline extraction; dmb, direct methylene blue; dx DEAT, derivatization of 2-amino-5-N,N-diethylamino toluene to thionine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); dx MBB, derivatization of monobromobimane to fluorescent sulfide derivative; dx PFBBr, derivatization of pentafluorobenzyl bromide to bis(pentafluorobenzyl)sulfide; dx phen, derivatization of p-phenylenediamine to thionine; d.m., paper is describing a new method for measuring sulfide in blood or plasma; e.c.d., electrochemical detector; GC-CL, gas chromatography-chemiluminescence sulfur detector; GC-ECD, gas chromatography-electron capture detector; GC-MS, gas chromatography-mass spectrometry; g.d., gas dialysis; HPLC, high performance liquid chromatography; imb, indirect methylene blue; ion chrom, ion chromatography; ion int rev ph HPLC, ion-interaction reversed-phase HPLC; ISE, sulfide ion-selective electrode; microdist, microdistillation; N/D, not detected; none, no reference given for method and not describing new method; SA, Swiss Albino; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; Sw. alb., Swiss albino; WKY, Wistar-Kyoto.

Superscripts: a, plasma was ultrafiltered for 15 min before assay; b, let blood stand for 2 h before taking serum; c, blood came from patients with non-fatal H₂S poisoning; d, cecum was inflated with H₂S gas during blood draw; e, six plasma H₂S concentrations of 2-6 µM were listed in a table while values 42, 47, and 52 µM were reported in text. The reason for the difference was not clear; f, add NaOH to sample prior to acidification, which could lead to desulfuration of protein as in direct ISE method.
APPENDIX II, LIST OF PHYSIOLOGIC AND PHARMACOLOGIC ACTIONS PROPOSED FOR H$_2$S

**Calcium:**
- stimulates T-type Ca$^{2+}$ channel currents (Kawabata et al., 2007)
- stimulates L-type Ca channel in cerebellar granule neurons (Garcia-Bereguiain et al., 2008)
- inhibits L-type Ca channel in rat cardiomyocytes (Sun et al., 2008)

**Potassium:**
- directly acts on K$_{ATP}$ channels in rat mesenteric artery SMC (Tang et al., 2005)
- inhibits large-conductance calcium-sensitive K channels, BK$_{Ca}$ (Telezhkin et al., 2009)
- modulates charybdotoxin/apamin-sensitive K channels in endothelium (Zhao & Wang, 2002; Cheng et al., 2004)

**Chloride:**
- upregulates GABA$_B$R2 expression (Han et al., 2005)
- inhibits Cl$^-$ channels from rat heart lysosomes (Malekova et al., 2009)
- activates Cl$^-$/HCO$_3$ exchanger in vascular smc (Lee et al., 2007)

**Cation:**
- enhances NMDA receptor current via cAMP-PKA (Abe and Kimura, 1996; Kimura, 2000)
- stimulates transient receptor potential vanilloid 1 (TRPV1) in guinea-pig airway neurogenic inflammation (Trevisani et al., 2005)
- inhibits renal Na$^+$/K$^+$/2Cl$^-$ cotransporter (Xia et al., 2009)

**NO:**
- H$_2$S releases NO from nitrosothiols (Ondrias et al., 2008)
- directly inhibits eNOS (Kubo et al., 2007)

**Other:**
- inhibits c-Jun N-terminal kinase (decreases apoptosis) (Shi et al., 2009)
- stimulates γ-GCS (Kimura & Kimura, 2004)
- stimulates cystine/glutamate antiporter (Kimura & Kimura, 2004)
- stimulates tyrosine kinases (Umemura & Kimura, 2007)
- H$_2$S stimulates HIF in *C. elegans* independent of hypoxia (Budde & Roth, 2009)
- increases Akt phosphorylation thus angiogenesis (Cai et al., 2007)
- decreases Akt phosphorylation (Geng et al., 2007)
- directly inhibits angiotensin converting enzyme in HUVEC by interfering with Zn$^{2+}$ (Laggner et al., 2007)
- decreases ICAM-1 expression by inhibiting activation of NF-κβ (Wang et al., 2009)
APPENDIX III, LIST OF ABBREVIATIONS

3MST – 3-mercaptopyruvate sulfurtransferase
AHSS – amperometric hydrogen sulfide sensor
akg - α-ketoglutarate
AOAA – aminooxyacetic acid
BCA - β-cyanoalanine
BSA – bovine serum albumin
cAMP – cyclic adenosinemonophosphate
CBS – cystathionine beta synthase
cGMP – cyclic guanosinemonophosphate
CL – cysteine lyase
CLP – cecal ligation and puncture
CSE – cystathionine gamma lyase
cys – cysteine
DMEM – Dulbecco’s modified Eagle’s medium
DTNB - 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)
DTPA – diethylenetriaminepentacetic acid
DTT - dithiothreitol
EC_{50} – effective concentration, concentration at which 50% response is achieved
ED – effective dose
EDTA – ethylenediaminetetraacetic acid
eNOS – endothelial nitric oxide synthase
FBS – fetal bovine serum
FiO2 – fractional inspired oxygen
FMA – fluorescein mercuric acetate
GABA – gamma aminobutyric acid
GSH – reduced glutathione
GSSG – oxidized glutathione
HA - hydroxylamine
HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HSD – Harlen-Sprague-Dawley
IP - intraperitoneal
IP3 – inositol 1,4,5-triphosphate
ISE – sulfide-ion selective electrode
LC_{50} – lethal concentration, concentration at which 50% of subjects die
L-NAME – L-nitro arginine methyl ester
LPS - lipopolysaccharide
LW – Lubund-Wistar
MI – myocardial infarction
MTMS – methyltrimethoxysilane
ND – not detected
NMDA – n-methyl-D-aspartate
NOS3 – nitric oxide synthase 3
P$_{50}$ – partial pressure at 50% of maximal response
PaO$_2$ – arterial partial pressure of oxygen
PASMC – pulmonary artery smooth muscle cells
PBS – phosphate buffered saline
PEBBLE – photonic explorer for bioanalysis with biologically localized embedding
PLP – pyridoxal phosphate
PPG – propargylglycine
ppm – parts per million
PTMS – phenyltrimethoxysilane
PVDF – polyvinylene difluoride
RBC – red blood cell
RCR – respiratory coupling (or control) ratio
ROS – reactive oxygen species
SAM – S-adenosylmethionine
SAOB – sulfide antioxidant buffer
succ - succinylcholine
SE – standard error
SD – standard deviation
SQR – sulfide quinone oxidoreductase
TCA – trichloroacetic acid
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