The Therapeutic Potential of Hydrogen Sulfide: Separating Hype from Hope

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**Running Head**: Therapeutic potential of H$_2$S

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ABSTRACT

Hydrogen sulfide (H₂S) has become the hot new signaling molecule that seemingly affects all organ systems and biological processes in which it has been investigated. It has also been shown to have both pro- and anti-inflammatory actions and pro- and anti-apoptotic effects and has even been reported to induce a hypometabolic state (suspended animation) in a few vertebrates. The exuberance over potential clinical applications of natural and synthetic H₂S “donating” compounds is understandable and a number of these function-targeted drugs have been developed and show clinical promise. However, the concentration of H₂S in tissues and blood, and the intrinsic factors that affect these levels, have not been resolved and it is imperative to address these points in order to distinguish between the physiological, pharmacological and toxicological effects of this molecule. This review will provide an overview of H₂S metabolism, a summary of many of its reported “physiological” actions and it will discuss the recent development of a number of H₂S “donating drugs that show clinical potential. It will also examine some of the misconceptions of H₂S chemistry that have appeared in the literature and attempt to realign the definition of “physiological” H₂S concentrations upon which much of this exuberance has been established.

Keywords: hydrogen sulfide donating drugs, vasoactivity, ischemia reperfusion injury, sulfur cycle, gasotransmitter
Abbreviations:

ADT-OH, Anethole trithione hydroxide
BCA, β-cyanoalanine
DADS, diallyl disulfide
DATS, diallyl trisulfide
CA, carbonic anhydrase
CAT, cysteine aminotransferase
CBS, cystathionine β-synthase
CDO, cysteine dioxygenase
CLY, cysteine lyase
CO, carbon monoxide
COS, carbonyl sulfide
Csa, cysteinesulfinate
CSD, cysteinesulfinate decarboxylase
CSE, cystathionine γ-ligase
Cys, cysteine
EDRF, endothelium-derived relaxing factor
MBA, monobromodimane–based assay
Hcy, homocysteine
MST, 3-mercaptopyruvate sulfurtransferase
NSAID, nonsteroidal anti-inflammatory drug
NO, nitric oxide
NOS, nitric oxide synthase
PPG, propargyl glycine
SDO sulfur dioxygenase
SQR, sulfide:quinone oxidoreductase
ST sulfur transferase
Introduction

The initial discovery by Hideo Kimura’s group that hydrogen sulfide (H$_2$S$^{1}$) was a biologically relevant signaling molecule (reviewed in 74) has heightened interest in the physiology and pharmacology of gaseous mediators. Unlike the first gaseous signaling molecule, nitric oxide (NO), whose introduction was met with initial skepticism, H$_2$S has more or less been enthusiastically embraced by the scientific community and there has been considerable effort to expeditiously imbue this obnoxious smelling gas into medical applications. This wave of exuberance has re-heightened interest in the dietary sources of H$_2$S and it has spawned the development of a number of H$_2$S-“donating” drugs, many of which are in various stages of clinical trials. However, is becoming increasingly evident that there is still much to be learned about the basic properties of H$_2$S measurement, metabolism and signaling mechanisms. This review will provide an overview of the effects of H$_2$S on physiological systems, summarize the new H$_2$S donating drugs that are showing clinical potential, and take a critical look at the some of the uncertainties surrounding H$_2$S chemistry and tissue concentrations that remain.

Hydrogen sulfide as a toxic gas

The toxic effects of H$_2$S have been known for centuries and it remains second only to carbon monoxide as the most common cause of gas-related fatalities in the workplace (46, 190). H$_2$S has even gained notoriety in a recent spate of 220 suicide cases in less than three months in Japan (107). Less is known of the effects of low-level ambient H$_2$S that are often associated with sewage plants, waste lagoons, natural gas/oil wells and oil refineries as well as a variety of other

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$^{1}$ Unless otherwise noted, H$_2$S refers to the sum of dissolved H$_2$S gas and HS$, often referred to as “sulfide”. At physiological pH, $S^{2-}$ is assumed to be negligible.
industrial applications. Recent studies on residents of Southeastern New Mexico exposed to
these environments have shown positive correlations with H₂S exposure and impaired
neurobehavioral functions when compared to controls (73). This suggests that even “therapeutic”
use of H₂S is not without potential hazards. Thresholds for the major effects of H₂S exposure are
shown in Table 1.

The inhibitory effects of H₂S on mitochondrial cytochrome c-oxidase have been well
categorized and this is generally assumed to be the focus of H₂S toxicity (34). However, the
clinical presentation of poisoning by H₂S and cyanide, another well known inhibitor of oxidative
phosphorylation that also inhibits cytochrome c-oxidase, are so distinct as to suggest different
modes of toxicity (46). Another rather unusual feature of H₂S toxicity is an extremely steep
dose-effect response. Early studies in dogs (47) and other mammals (38, 25) and more recent
anecdotal information from human cases (46) have shown that H₂S toxicity is closely correlated
with H₂S concentration and considerably less dependent upon the duration of exposure. This
suggests that animals can rapidly metabolize H₂S up to a critical level and, as a corollary, this
efficient metabolic capacity should keep free H₂S at very low levels. These studies should, but
have not often, raised questions regarding “physiological” concentrations of H₂S in tissues and
blood. This point is discussed in detail in a later section.

Hydrogen sulfide biosynthesis and metabolism.

Biosynthesis.

Much of the metabolism of sulfides, including H₂S, passes through cysteine (Cys)
metabolism (Fig. 1). Cysteine can be oxidized to cysteinesulfinate (Csa) or it can be desulfurated
by reducing reactions that generate either \( \text{H}_2\text{S} \) or sulfane sulfur (a persulfide; 149). In the oxidative, and generally assumed catabolic pathway for cysteine, cysteine dioxygenase (CDO) catalyzes the addition of molecular oxygen to cysteine producing \( \text{Csa} \), which may be further oxidized to sulfite or taurine (149). As perhaps a general indication of a broad-spectrum of sulfur-mediated effects on biological systems, both \( \text{Csa} \) and its metabolites have themselves been shown to affect a variety of physiological processes (68, 100). CDO is found in liver, adipose, intestine, pancreas and kidney. Because activity of CDO is highly regulated by dietary cysteine, CDO is a, if not the primary, regulator of cysteine availability in vivo. By oxidizing excess and presumably toxic cysteine, CDO provides a constant and low-level background of cysteine for \( \text{H}_2\text{S} \) and sulfane sulfur biosynthesis. This may be important in preventing excessive \( \text{H}_2\text{S} \) production (33).

\( \text{H}_2\text{S} \) can be produced from cysteine via a variety of biochemical pathways. Early studies indicated that cystathionine \( \beta \)-synthase (CBS) was the predominant enzymatic pathway for \( \text{H}_2\text{S} \) production in the brain, whereas cystathionine \( \gamma \)-lyase (CSE aka CGL) was responsible for \( \text{H}_2\text{S} \) production in the vasculature (75). Recent studies have shown that cystathionine \( \beta \)-synthase (CBS) is present in the endothelium and two enzymes acting in tandem, cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (MST), are also present in vascular endothelium and brain, whereas MST, but not CAT, is found in vascular smooth muscle (75, 119). CAT transfers the amine group from cysteine to an acceptor such as \( \alpha \)-ketoglutarate resulting in 3 mercaptopyruvate which is then de-sulfurated by MST. In addition to \( \text{H}_2\text{S} \), reduced sulfur in the form of sulfane sulfur can also be generated and in fact sulfane sulfur appears to be the only product of the CAT-MST pathway (66). Kimura’s group found relatively
high levels of CAT-MST in the brain and they proposed that this is a major pathway for H₂S production in the brain but they also suggested that the H₂S is immediately “stored” as sulfane sulfur, the latter serving as a less labile form of H₂S that may be readily accessible during appropriate physiological conditions (60, 141). However, reducing conditions and an alkaline environment are necessary for cleavage of this RS-S bond to form H₂S and because these conditions may not be routinely encountered intracellularly, the significance of the CAT/MST pathway in H₂S synthesis remains questionable. Both CBS and CSE have recently been shown to circulate in human plasma and to generate H₂S from cysteine or homocysteine plus cysteine (13). This has been proposed to not only reduce circulating homocysteine, but the H₂S generated may protect the endothelium from oxidative stress.

Both CBS and CSE are cytosolic, pyridoxyl 5'-phosphate-dependent, enzymes. CBS activity appears to be controlled by a number of factors. S-adenosylmethionine (AdoMet), is an allosteric activator of CBS and when AdoMet levels are low CBS activity decreases to direct sulfur flow through the transmethylation pathway thereby conserving methionine. Elevated AdoMet increases CBS activity to produce cysteine via the transsulfuration pathway (148). CBS contains a heme group which when it binds with carbon monoxide (CO) inhibits the enzyme. CBS is also inhibited by reducing conditions, but contrary to a number of earlier reports neither nitric oxide (NO) nor calmodulin appear to be physiological regulators of CBS activity (8).

Using physiologically relevant substrate concentrations and kinetic simulations, Banerjee’s group (cf; 23, 66, 143) concluded that; 1) H₂S generation from cysteine is primarily catalyzed by CSE, 2) H₂S production by CBS is through condensation of cysteine and homocysteine and depending on the level of AdoMet activation this may account for 25-70% of
the H$_2$S generated under resting conditions, 3) H$_2$S biosynthesis can occur independent of
cysteine; condensation of two molecules of homocysteine, catalyzed by CSE, yields
homolanthionine and H$_2$S, and may account for as much as 30% of the total H$_2$S biosynthesis, 4)
CSE activity is substantially increased by elevated homocysteine, whereas CBS activity is
unaffected. Condensation of two homocysteine molecules, along with the condensation of
homocysteine and cysteine, appear to be important clearance pathways in
hyperhomocysteinemia. It has been proposed that during severely elevated homocysteine (200
μM), as seen in hyperhomocysteinemia, α,γ-elimination and γ-replacement of homocysteine,
catalyzed by CSE, may produce excessive amounts of H$_2$S and thereby contribute to the
cardiovascular pathology associated with this condition (23).

Commonly used inhibitors of CSE include propargyl glycine (PPG) and β-cyanoalanine
(BCA). Aminooxyacetate (AOA) is commonly used to inhibit CBS and hydroxylamine (HA) to
inhibit both enzymes (although a number of studies erroneously claim this is a specific inhibitor
of CBS). Unfortunately, none of these inhibitors are specific for sulfur metabolism and H$_2$S
production, furthermore, they are often poorly absorbed by tissues (153).

Other potential biosynthetic pathways.

There are numerous other potential metabolic pathways for H$_2$S generation that have
been described in invertebrates (Fig. 1; 65) but these have not been systematically evaluated in
mammalian tissues. The resurgent interest in H$_2$S will undoubtedly lead to reevaluation of these
heretofore overlooked biosynthetic pathways and identification of novel ones. Indeed, the
literature is replete with studies that show that many of the biological effects produced by H$_2$S
can also be affected by a variety of other sulfur donating molecules. One potentially novel pathway that needs to be investigated is H$_2$S production from carbonyl sulfide (COS; chemical structure: O=C=S). Like H$_2$S, COS is a gas that has both natural (volcanoes, hot springs, oils and trees) and man-made (biomass and fossil fuel consumption, wastewater treatment, etc.) origins and it is the most prevalent sulfur gas in the atmosphere (152). COS is the only volatile sulfur that is increased in exhaled air of patients with cystic fibrosis (69) or of lung transplant patients during the acute rejection phase (150). COS is also exhaled by patients with chronic liver disease (135). COS has been demonstrated to be produced by porcine coronary arteries in vitro and the rate of COS production is enhanced by stimulating the vessels with acetylcholine or the calcium ionophore, A23187 (7). In solution, COS slowly decomposes to H$_2$S but this reaction is greatly accelerated by the enzyme carbonic anhydrase. In fact, CO$_2$ and COS may be the primary substrates of this enzyme (134). Whether or not the biosynthesis of COS is related to H$_2$S production and subsequent signaling events remains to be determined.

**Metabolism (inactivation).**

Oxidation of H$_2$S occurs in the mitochondria (53). As shown in Fig. 2, two membrane-bound sulfide:quinone oxidoreductases (SQR) oxidize sulfide to the level of elemental sulfur, simultaneously reducing cysteine disulfide, and resulting in formation of persulfide groups at one of the SQR cysteines (SQR-SSH). Sulfur dioxygenase (SDO) then oxidizes one of the persulfides to sulfite (H$_2$SO$_3$), consuming molecular oxygen and water in the process. Sulfur from the second persulfide is transferred from the SQR to sulfite by sulfur transferase (ST) producing thiosulfate (H$_2$S$_2$O$_3$). Most thiosulfate is further metabolized to
sulfate by thiosulfate reductase and sulfite oxidase. Electrons from H$_2$S are fed into the respiratory chain via the quinone pool (Q), and finally transferred to oxygen at complex IV. Oxygen consumption is obligatory during H$_2$S metabolism and one mole of oxygen is consumed for every mole of H$_2$S oxidized along the electron transport chain (53). Oxidation of H$_2$S to thiosulfate requires additional oxygen at the level of SDO resulting in a net utilization of 1.5 moles of oxygen per mole of H$_2$S (or 0.75 moles of O$_2$ per mole H$_2$S; 82). Metabolism of H$_2$S through SQR appears ubiquitous in tissues, a notable exception being brain (82). It is important to note that sulfide oxidation in the mitochondria appears to take priority over oxidation of other carbon-based substrates ensuring its efficient removal (24). This plus the fact that the capacity of cells to oxidize sulfide appears to be considerably greater than the estimated rate of sulfide production (24) ensures that intracellular H$_2$S concentrations are very low. Interestingly, the statin, atorvastatin, increases H$_2$S production in perivascular adipose tissue by producing coenzyme Q$_9$ deficiency and thereby inhibiting mitochondrial oxidation (189).

The relationship between H$_2$S and O$_2$ consumption is classical hormesis; at low concentrations H$_2$S stimulates oxygen consumption (and may even result in net ATP production), whereas it is inhibited by elevated H$_2$S. This was originally shown in invertebrates and lower vertebrates and more recently demonstrated in the mammalian colon (45). At higher concentrations H$_2$S inhibits the respiratory chain by directly inhibiting cytochrome c-oxidase (24). The exact H$_2$S concentration at which this occurs is unclear, purified cytochrome c-oxidase is inhibited by <1 μM H$_2$S, whereas progressively greater (2-3 orders of magnitude) higher H$_2$S concentrations are needed to inhibit the enzyme in intact mitochondria and then whole cells. Cytochrome c-oxidase is half maximally inhibited by ~ 20 μM H$_2$S and may not be fully
inhibited until \( \text{H}_2\text{S} \) concentrations reach 40-50 \( \mu \text{M} \) \((6, 24)\). This may reflect diffusion limitation as the enzyme becomes further removed from the exogenously administered \( \text{H}_2\text{S} \). It also should provide a cautionary note to interpreting studies that routinely employ 100 \( \mu \text{M-1 mM} \) \( \text{H}_2\text{S} \) in order to demonstrate a “physiological” effect. The converse, i.e., the effect of \( \text{O}_2 \) on \( \text{H}_2\text{S} \) consumption, is discussed in the section “\( \text{H}_2\text{S} \) and oxygen sensing”.

**\( \text{H}_2\text{S} \) Biology**

Interest in \( \text{H}_2\text{S} \) biology has spawned nearly as many reviews (at latest count 32 in 2010 alone) as original articles. Reviews have even appeared where, at the time, the effects of \( \text{H}_2\text{S} \) on a particular system were unknown \((87, 196)\). The following sections provide a brief overview of \( \text{H}_2\text{S} \) biology. For further details the reader is referred to a few of the most recent reviews following each section.

**\( \text{H}_2\text{S} \) and the nervous system.**

Potentiation of the \( \text{N}-\text{methyl-D-aspartate} \) (NMDA) receptor and the resultant alteration of long-term potentiation (LTP) in the hippocampus was the first biological effect ascribed to \( \text{H}_2\text{S} \) \((1)\). Not long thereafter it was noted that patients with Down’s syndrome had elevated concentrations of \( \text{H}_2\text{S} \) in cerebral spinal fluid. This would be predicted from the fact that chromosome 21 encodes CBS (which may be the major \( \text{H}_2\text{S} \)-producing enzyme in the brain) and is over-expressed in these patients \((70)\). It has also been suggested that deficiencies in \( \text{H}_2\text{S} \) biosynthesis are associated with Alzheimer’s disease \((37, \text{reviewed in 130})\) and that exogenous \( \text{H}_2\text{S} \) may have therapeutic potential by reducing amyloid beta protein plaques \((201)\). \( \text{H}_2\text{S} \) has...
been proposed to modulate nociception (40, 144), induce μ opioid receptor-dependent analgesia (30), prevent neurodegeneration and movement disorders in mouse models of Parkinson’s disease (55, 72) and may reduce the stress response of the hypothalamic-pituitary-adrenal axis (102). It has also been proposed to antagonize homocysteine induced neurotoxicity (162).

The protective effects of H2S have been demonstrated in a number of neurological systems. H2S has been shown to protect neurons against hypoxic injury (165), inhibit hypochlorous acid-mediated oxidative damage (183) and increase glutathione production and suppress oxidative stress in mitochondria (76). Conversely, H2S has been shown to mediate cerebral ischemic damage (129) and produce vanilloid receptor 1-mediated neurogenic inflammation in airways (170).

H2S increases cAMP production in neurons and subsequent activation of protein kinase A (PKA) may account portion of the LTP. Other functions of H2S include upregulation of γ-amino butyric acid (GABA) B receptor and neuronal hyperpolarization via KATP channel activation and induction of calcium waves in astrocytes (130), regulation of intracellular pH in glial cells (98), and the above mentioned increase in glutathione production. Recent reviews: 56, 160, 144, 130.

H2S and the gastrointestinal system.

The initial interest in H2S in the gastrointestinal (G.I.) system stemmed from the well-known production of H2S by sulfate-reducing bacteria in the colon and the presumed need to protect tissues from this toxic molecule (133). Today more is known about the effects of H2S in the colon than any other segment of the G.I. tract, however, anti-inflammatory actions of H2S
in the stomach appear to be of important therapeutic value and other areas have received increased attention as well.

H₂S is synthesized in the stomach, jejunum, ileum and colon. CSE immunoreactivity is diffusely distributed throughout the gastrointestinal tract most likely due to its association with the vasculature, whereas CBS staining is predominantly in muscular as mucosa cell mucosa and laminar propria but not associated with goblet, crypt and epithelial cells (105).

H₂S relaxes smooth muscle in the stomach (28) intestine (113) and colon (29). The mechanisms of H₂S on G.I. motility have not been fully resolved and in most instances we are merely left with a list of factors that do not affect motility. In the stomach H₂S acts partly via activation of myosin light chain phosphatase (28), in the colon the effects of H₂S are independent of intracellular calcium and not mediated through known K⁺ channels, myosin light chain phosphatase or Rho kinase (29) and in the ileum H₂S relaxation is independent of extrinsic or enteric nerves, NO, K⁺ATP and K⁺Ca⁺ channels (113). H₂S inhibits pacemaker activity of mouse small intestine interstitial cells of Cajal by modulating intracellular calcium through mechanisms independent of K⁺ channels (122). Proliferation of these interstitial cells is also stimulated by H₂S which acts via phosphorylation of AKT protein kinase (57). H₂S stimulates chloride secretion in the intestine by targeting vanilloid receptors (TRPV1) on afferent nerves which in turn activate cholinergic secretomotor neurons via release of substance P (79).

H₂S has both anti-inflammatory and inflammatory effects in the G.I. tract, however, the former is perhaps better characterized and appears to be of therapeutic value. In the colon, H₂S is anti-inflammatory and enhances ulcer healing independent of nitric oxide synthase (NOS) and K⁺ATP channel involvement (176). H₂S production is increased in experimental models of colitis.
and H2S protects against and promotes resolution of this colitis (177). However, H2S modulates the expression of genes involved in cell-cycle progression and may trigger both inflammatory and DNA repair processes which may contribute to colorectal cancer (5).

In the pancreas H2S is a mediator of inflammatory caerulein-induced pancreatitis (17, 158, 159). H2S acts through ICAM-1 expression and stimulates neutrophil adhesion through the NF-κB and Src-family kinases (157). However, H2S has also been shown to protect pancreatic β cells from oxidative stress (164).

Inhibition of CSE, which is found in both hepatocytes and the bile duct, stimulates biliary bicarbonate secretion, whereas exogenous H2S inhibits it (39). Bile acids increase liver CSE expression via activation of the farnesoid X receptor, the resultant H2S production is proposed to maintain vasodilation and minimize the chance for portal hypertension (131).

Recent reviews: 64, 71, 96, 106, 133, 175.

**H2S and the cardiovascular system.**

Collectively, the involvement of H2S on heart and blood vessel physiology has received more attention than any other organ system, even though the therapeutic applications of H2S are less evident.

The vasodilatory effects of H2S on systemic blood vessels were the first cardiovascular effects of this transmitter described (54). This has been confirmed repeatedly and even observed in pulmonary arteries of diving mammals (119). H2S-induced relaxation appears to depend on extracellular Ca2+ (203), and although KATP channels, are frequently assumed to mediate the H2S relaxation (63, 86, 203, 204), this mechanism typically accounts for no more
than half of the relaxation in most vessels. In some animals, such as the mouse, KATP channels are not involved at all in the response. H$_2$S may also signal via other pathways such as activation of adenylate cyclase, which in turn inhibits superoxide formation, NADPH oxidase and Rac$_1$ activity (112), it may produce intracellular acidosis and alter intracellular redox status, stimulate an anion exchanger (97) or operate through Ca$^{2+}$-dependent K$^+$ (K$_{Ca}$) channels (77, 161, 206). Relaxation of rat aorta by exogenous H$_2$S does not depend on vascular prostaglandin synthesis, protein kinase C, or cAMP, nor does it involve superoxide or H$_2$O$_2$ production (77, 78, 204). Observations that H$_2$S sulfhydrates and may regulate biological activity of numerous proteins including actin (109) suggests that additional key steps in H$_2$S-mediated vascular signaling are soon to be unraveled. However, even this mechanism has been questioned based on the seemingly non-selectivity and promiscuity of this process (96) and the suggestion that for this to occur the cysteine residues must be in the oxidized state and these are rare in the reducing intracellular environment (66). H$_2$S may also indirectly relax blood vessels in vivo through its ability to inhibit angiotensin converting enzyme and thus prevent formation of the vasoconstrictor angiotensin II.

Recent evidence has turned to H$_2$S as the elusive endothelium-derived hyperpolarization factor, the third endothelium-derived relaxing factor (EDRF) that, along with NO and prostacyclin, signals vasodilation (180). Crosstalk between H$_2$S, NO and CO has suggested to contribute to vasoactivity and, although CO inhibits CBS (8), interactions between H$_2$S and NO are far from resolved. NO production has been shown to be directly inhibited by H$_2$S (81) or indirectly stimulated by it through activating nuclear factor (NF)-κB which activates the extracellular regulated kinase 1/2 (ERK1/2) which in turn activates iNOS (62). H$_2$S relaxations
have been reported to be independent of NO synthesis or cGMP activation (77, 78, 203). As described above, NO does not appear to directly affect H2S production (8). There is also evidence that H2S and NO may form a simple S-nitrosothiol with vasoactive properties of its own (184). Reports of H2S-mediated vasoconstrictory responses in mammalian systemic vessels are less common, and many of these show an endothelium-dependent effect that has been attributed to H2S inactivation of NO. Low concentrations of H2S (<200 μM) produce endothelium-dependent contraction of human internal mammary arteries and rat and mouse aortas (2, 81, 181) and low-dose H2S infusion increases blood pressure in the rat (2). These contractions have been proposed to result from H2S inactivation of endothelial NO via production of an inactive nitrosothiol (2, 181), whereas Kubu et al. (81)) showed that H2S directly inhibited NO production. Other studies suggest that H2S may have direct, albeit modest, constrictory effects on systemic vascular smooth muscle. Lim (95) observed 1 μM H2S contractions of rat aortas that were partially independent of both the endothelium and KATP channels and due in part to down-regulation of cAMP. Direct H2S-mediated vasoconstriction has been demonstrated in systemic vessels of nonmammalian vertebrates and H2S contracts pulmonary vessels in terrestrial mammals in response to hypoxia (32, 117, 118).

H2S has a variety of other effects on the vasculature that are not directly vasoactive. At times the findings are contradictory, but nevertheless, many are suggestive of therapeutic potential. H2S has been shown to be both pro- and anti-inflammatory, reduce leukocyte adhesion, inhibit platelet aggregation and although it is pro-angiogenic, it reduce deleterious vascular remodeling that often accompanies vascular damage (35, 89, 155). H2S is not only a
mild antioxidant, but it also stimulates cysteine uptake and synthesis of glutathione. H₂S has been implicated in hypotension associated with septic and hypovolemic shock and inappropriate H₂S regulation of insulin secretion in type II diabetes may contribute to macro- and micro-vascular pathologies (85). Inhibition of plasma renin activity by H₂S is antihypertensive in renin-dependent hypertensive rats (99) and can potentially augment the depressor effect of H₂S vasodilation.

While H₂S has been shown to have negative inotropic and chronotropic effects on the heart (207), most interest has centered around its cardioprotective abilities. Numerous studies have shown that transient application of H₂S or H₂S donors can mimic hypoxic pre- and post-conditioning and that increased endogenous H₂S biosynthesis can also protect the heart from ischemia/reperfusion injury (reviewed in; 35, 83, 156). Furthermore, the potential for H₂S-mediated protection from ischemia/reperfusion injury has been demonstrated in a number of extracardiac organs including the kidney (171), which presumably offsets the reduction in endogenous H₂S-production (192), liver and small intestine (52, 198), skeletal muscle (49, 51) and cellular components of cutaneous tissue (50). As in the vasculature, H₂S has been proposed to combine with NO to produce a nitrosothiol with inotropic properties (195, 194).

Recent reviews: 11, 15, 36, 83, 114, 155, 156, 172, 180, 207.

\[H₂S and the respiratory system.\]

Much of the focus of H₂S activity in the lung has focused on pulmonary blood flow and pulmonary vascular resistance. Increasing pulmonary blood flow in rats via a aortocaval shunt decreases CSE mRNA and CSE-mediated H₂S production (140). During chronic hypoxia and the
associated pulmonary hypertension, plasma and lung tissue production of H$_2$S is decreased (182, 199, 202) and CSE activity is suppressed (199). Hypoxic pulmonary hypertension is further increased after CSE inhibition with PPG (199, 202), whereas exogenous H$_2$S reduces pulmonary arterial pressure (182, 199) but (surprisingly) this does not affect aortic pressure (199). Exogenous H$_2$S also decreases tissue GSSG and increases total antioxidant capacity (182).

H$_2$S paradoxically constricts isolated resistance pulmonary arterioles in terrestrial mammals but dilates those of diving mammals (sea lions) which is consistent with the response of these vessels to hypoxia and the specific needs of the animal (119). H$_2$S also relaxes pre-contracted mouse bronchial smooth muscle via a mechanism that is independent of KATP channels, soluble guanylyl cyclase, cyclooxygenases 1 and 2, tachykinins (80).

Plasma H$_2$S is reported to decrease in rats with oelic acid-induced lung injury; exogenous H$_2$S increases arterial PaO$_2$, decreases pulmonary edema and infiltration of polymorphonuclear cells, decreases IL-6 and IL-8 but increases IL10 suggesting endogenous H$_2$S production is decreased in this model of lung injury (93). CSE expression in airway and vascular smooth muscle decreases in olvalbumin induced lung asthma whereas exogenous H$_2$S alleviates inflammation, restores expiratory flow and attenuates iNOS activation (22). Ventilator induced lung injury also enhances the inflammatory response which is reversed by exogenous H$_2$S (3). These studies suggest H$_2$S is anti-inflammatory and anti-remodeling in a variety of lung pathologies in addition to hypoxia.

Recent reviews: 120.

H$_2$S and the kidney.
H₂S affects both the renal tubule and vasculature. H₂S is produced in the kidney by combined actions of CBS and CSE (191). Simultaneous administration of AOA and PPG (but neither independently) decreases GFR, sodium and potassium excretion, whereas these are increased by infusion of either H₂S or Cys (191). In the two-kidney-one-clip rat model of renal vascular hypertension, exogenous H₂S decreases blood pressure, decreases plasma renin activity and angiotensin II concentration (but it does not affect plasma angiotensin converting enzyme activity), and it inhibits upregulation of renin mRNA (99). In a genetic model of hyperhomocysteinemia, H₂S production is down regulated, GFR decreases, glomerular inflammation increases, and these effects that can be reversed by exogenous H₂S (138). In a mouse model of diabetic nephropathy induced by streptozotocin, plasma and renal cortex H₂S decrease, TGF-β1 and collagen IV increase, and these changes are prevented by exogenous H₂S (197). Upregulation of TGF-β1 and collagen IV and reduced CSE expression produced by high glucose in culture mesangial cells are also prevented by exogenous H₂S (197). In human patients, hemodialysis appears to lower plasma H₂S (125).

Recent reviews: 14.

H₂S and reproduction.

Although CBS has been identified in Leydig, Sertoli, and germ cells, and CSE has been found in Sertoli cells and germ cells in the rat testis (151), most of the attention has been focused on the vasodilatory properties of H₂S in the corpus cavernosum and the potential for H₂S therapy in erectile dysfunction (26, 146). Human and rat vas deferens smooth muscle contains both CBS and CSE and is relaxed by H₂S (88). H₂S has also been shown to be synthesized by the rat
uterus, fetal membranes and placenta as well as human placenta. CBS and CSE were identified in all rat intrauterine tissues as well as in human placenta myometrium, amnion and chorion (123). H2S also produces dilation in vaginal and clitoral cavernosal muscle strips in the rabbit (147). However, the role of H2S in reproduction per se is unknown.

H2S interactions with heme proteins.

Because of the known interactions of NO and CO with iron centers in a variety of heme proteins, the ability of H2S to reduce methemoglobin, the estimation that acid labile H2S could be released from a variety of cytochromes, and the inhibitory effect of H2S on cytochrome c-oxidase, it was only natural to assume that H2S would serve some physiological function in heme proteins. Although it has been observed that a modified hemoglobin, hemoglobin I (HbI) does indeed serve a physiological function in sulfide transport in the clam (Lucina pectinata), there does not seem to be an analogous activity in mammals. Relatively high H2S concentrations (3:1, H2S: hemoglobin) favor formation of sulfhemoglobin and sulfmyoglobin which may lower the oxygen affinity by 135 and 2,500 fold, respectively but this does not appear to be a physiological process because is doubtful that H2S concentrations even approach these levels in vivo. While H2S concentrations may increase during toxic exposure, the adverse effect of sulfhemoglobin formation will be somewhat offset by a concomitant H2S-induced right shift in the oxyhemoglobin curve. In most cases, sulfhemoglobinemia toxicity is well tolerated and resolved by red blood cell replacement. Sulfheme formation requires a histidine residue in the heme environment. Such a histidine is lacking in cytochrome c which therefore does not form a sulfheme when exposed to H2S (reviewed in 127). H2S has recently been shown to form
sulpheme with human neuroglobin, which also appears to depend on a histidine residue (18).

However, this reaction was produced by first forming ferric (Fe$^{3+}$) neuroglobin and the physiological significance of this oxidized form is unknown.

Recent reviews: 127.

__H$_2$S and oxygen sensing.\_

We initially proposed that H$_2$S metabolism serves as an intrinsic oxygen sensor in the vasculature (117) and these observations have been extended to include systemic and pulmonary vessels from a variety of vertebrates (117, 119), in fish gill chemoreceptive cells (118), urinary bladder (31) and the mammalian carotid body (124, 166, 167). Key in this hypothesis is the ability of tissues to rapidly consume H$_2$S in the presence of oxygen (Fig. 3A) and observations that the rate of H$_2$S metabolism is coupled to tissue or mitochondrial oxygen at physiologically relevant PO$_2$s (Fig. 3B). The relationship between oxygen consumption and H$_2$S production are considered in more detail below. Mechanisms of H$_2$S-mediated vasodilation were described previously in “H$_2$S and the cardiovascular system”. The mechanism of H$_2$S-mediated hypoxic vasoconstriction remains to be identified, although it may be similar to activation of type 1 glomus cells in the mammalian carotid body where H$_2$S inhibits large-conductance calcium-sensitive potassium (BK$_{Ca}$) channels (166, 167). Although never directly addressed in the literature, it seems likely that the hypoxia-induced increase in tissue H$_2$S also is the initial stimulus in pre-and post-conditioning effects associated with reperfusion injury and may also contribute to the pathology of reperfusion injury in unconditioned tissues.

Recent reviews: 14, 116, 118.
Recent studies showing that inhaled H\textsubscript{2}S can induce a “suspended animation-like state” in small mammals have heightened expectations of using H\textsubscript{2}S treatment clinically (4). Potential applications of this hypometabolic state, in addition to protection from ischemia/reperfusion injury described above, include organ preservation prior to transplantation, protective metabolic depression during bypass surgery or following severe trauma associated with shock, sepsis and acute lung injury (4). However, attempts to induce similar metabolic depression and protection in large mammals have produced conflicting results and clearly much needs to be done in this field prior to clinical applicability. Even in small rodents the metabolic effects of H\textsubscript{2}S remains unclear. Baumgart et al. (12) showed in small rodents that while H\textsubscript{2}S inhalation during hypothermia did not alter the hemodynamic and cardiac effects of hypothermia itself, it did improve mitochondrial respiration and they propose that this may be the benefit of exogenous H\textsubscript{2}S during hypothermia. Interestingly, the authors also found that the H\textsubscript{2}S exposure increased aerobic glucose utilization. This observation is difficult to reconcile with the metabolic studies of Bouillaud and Blachier (24) who showed a mitochondrial preference for sulfide oxidation over other carbon-based substrates see; “metabolism (inactivation)” above. Clearly more needs to be learned regarding the transition from mitochondrial oxidation of H\textsubscript{2}S as an energy source to the inhibitory effects of H\textsubscript{2}S on oxidative phosphorylation.

Recent Reviews: 4.

Other general reviews: 42, 48, 58, 67, 74, 75, 89, 90, 101, 110, 121, 136, 185, 200.
Disorders of H₂S metabolism

Relatively few clinical conditions are currently attributable to H₂S metabolism. Ethylmalonic encephalopathy is an autosomal recessive disorder characterized by early-onset encephalopathy, microangiopathy, chronic diarrhea and defective cytochtome c-oxidase (168). The gene ETHE1 encodes ETHE1, a mitochondrial dioxygenase, the absence of which appears to adversely affect mitochondrial H₂S oxidation resulting in elevated tissue H₂S and associated toxicity. Paradoxically, however, H₂S₃O₂ levels are also increased. Deficiencies in CBS activity result in hyperhomocysteinemia and the associated cardiovascular, ocular, neural and skeletal problems (103, 187). CSE deficiency produces cystathioninuria is secondarily associated with a wide range of diseases including diabetes insipidus, Down’s syndrome, neuoroblastoma, hepatoblastoma, and celiac disease, however, this is not associated with any overt clinical abnormalities (66). Mercaptolactate-cysteine disulfiduria is associated with MST deficiency and may or may not be associated with mental retardation (66).

Reviews: 66, 103, 137, 187.
H$_2$S “Donating” Drugs

An appreciation, if not understanding, of the health benefits of sulfur springs, garlic and cruciferous vegetables has known since ancient times. Recent evidence suggests that the common denominator in these folk remedies may be their ability to produce H$_2$S. Considerable effort is now underway to study and promote dietary intake of these sulfur-containing foods and in the synthesis of novel orally active compounds. The latter approach has proven especially effective when the H$_2$S donating compound is attached to another drug. A few of the more common H$_2$S donating drugs of this type are shown in Fig. 4. Additional drugs are described in recent reviews (20, 104) and the progress of these drugs in clinical trials can be accesses from the website “www.clinicaltrials.gov”. A list of patent applications on H$_2$S-releasing molecules and dosages can be found in Bannenberg and La Vieira (9).

Naturally occurring H$_2$S-donating drugs.

The best characterized naturally occurring H$_2$S-donating compound from garlic (Allium sativum) is allicin (diallyl thiosulfinate) which decomposes in water to a number of compounds. Two of these, diallyl disulfide (DADS) and diallyl trisulfide (DATS; Fig. 4A) are the most efficacious H$_2$S donors and readily vasodilate rat aortas (16). It should be noted garlic-mediated H$_2$S production by red blood cells and in buffer was determined under anoxic conditions and H$_2$S production by rat aorta was measured at PO$_2$ < 36 mmHg. How these reactions proceed under more physiological conditions (eg, PO$_2$) remains to be determined.

Sulforaphane (Fig. 4B), the isothiocyanate compound from broccoli (Brassica oleracea), protects vascular smooth muscle cells and endothelial cells from oxidative and inflammatory stress and suppresses angiogeniesis (61, 139, 208). It also protects hearts from ischemia.
reperfusion-induced injury (108). Sulforaphane also has neuroprotective and anti-inflammatory actions mediated in part through activation of heme oxygenase-1 (HO-1) and provides some protection against ischemia reperfusion injury, hemorrhage and serotonin-induced toxicity (19). Sulforaphane is rapidly absorbed by humans, reaching peak concentrations at 1 h and declining thereafter with a half-life of 1.8 hours (193). A related isothiocyanate compound, erucin, is found in high levels in rocket salad species (*Eruca sativa*) and produces a concentration-dependent induction of a number of cellular antioxidants and enzymes. It has not yet been determined if either sulforaphane or erucin are metabolized to H₂S, or if erucin has any beneficial effects on the cardiovascular system.

**Synthetic H₂S-donating drugs.**

A number of H₂S compounds have been synthesized with the intent of slowing the rate of H₂S release and thereby avoiding the transient H₂S surge characteristic of the sulfide salts. GYY4137 (morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate, Cayman Chemical; Fig. 4C; 92) is a water-soluble molecule that is reported to slowly (over 90 min) release H₂S in acidic phosphate buffer. When GYY4137 was injected intraperitoneally (ip) or intravenously (iv) into rats, plasma H₂S increased from control 33 μmol/l to ~80 μmol/l in 30 min and was still elevated (50 μmol/l) 3 hours later. By comparison, NaHS rapidly (within seconds) generated H₂S in buffer and did not affect plasma H₂S concentration when injected iv. GYY4137 produced a KATP channel-mediated relaxation of rat aortas and dilated the perfused kidney. In vivo, GYY4137 exhibits antihypertensive activity. Curiously, H₂S generation from either GYY4137 or NaHS in phosphate buffer was measured in real time with sensitive amperometric electrodes, whereas following injection of these compounds *in vivo*, plasma H₂S
was measured with an indirect and questionable methylene blue method (see section “separating hype from hope” below). Lawesson’s reagent (Fig. 4D), another H₂S donor has been used with some success as an anti-inflammatory drug in the stomach (176).

Stimulation of H₂S production and the augmentation of H₂S-like effects by exogenous cysteine are well known. Several cysteine analogues have been synthesized to mimic these effects such as S-propyl cysteine, S-allyl cysteine (in garlic) and S-propargyl cysteine and they exert the expected cardioprotective effects (178). S-propargyl cysteine has also shown promise in preventing cognitive impairment in a rat model of Alzheimer’s disease (44) and its structure is shown in (Fig. 4E). In a patent application (http://www.faqs.org/patents/app/20090036534) S-propargyl cysteine and S-allyl cysteine (50 mg/kg/day ip for 7 days) was reported to increase plasma H₂S in rats with myocardial injury from 34.7 to 91.6 and 61.1 μM, respectively.

Additional benefits have been realized by complexing H₂S-donors with other clinically efficacious drugs. The bulk of this work has successfully focused on blending the protective effects of H₂S with nonsteroidal anti-inflammatory drugs (NSAIDs) that of themselves often have adverse side effects in the gastrointestinal tract (174). Anethole trithione (ADT-OH Fig. 4F), a dithiolethione, is one of the most commonly used H₂S donors. This molecule has been combined with numerous NSAIDs including aspirin (ACS14, CTG-Pharma), diclofenac (ATB-337, Antibe Thrapeutics; ACS 15, S-diclofenac; Fig. 4G), indomethacin (ATB-343), mesalamine (ATB-429) and sulindac (S-sulindac). Dithioline derivatives of sildenafil (ACS6), valproate (ACS 2, S-valproate; Fig. 4H) and the anti-glaucoma drug latanoprost (ACS-67) have been developed as have other methanethiosulfonate derivatives of valproate (ACS 33, Fig. 4I and ACS 43, Fig. 4J; 20, 126). A number of H₂S-releasing molecules with potential antioxidant
and anti-inflammatory properties (Fig. 4.K-M) have been coupled to levodopa (84). A patent application for ADT-OH conjugated with the angiotensin AT1 receptor inhibitor losartan (H$_2$S-EXP 3714) has been reported (104) but efficacy studies have not been published.

The effects of H$_2$S-donating drugs are beginning to be examined. In cultured rat aortic smooth muscle cells S-diclofenac, but not diclofenac, dose-dependently inhibits cell proliferation and survival (10). S-diclofenac (47.2 $\mu$mol/kg ip) has no affect on blood pressure or heart rate over 180 min but down-regulates expression of genes encoding enzymes synthesizing nitric oxide, prostanoids, and H$_2$S. S-diclofenac also reduces plasma IL-1$\beta$/TNF-$\alpha$, elevates plasma IL-10 and increases plasma H$_2$S concentration from 25 to 37 $\mu$mol/l at 45 minutes and to 33 $\mu$mol/l 6 hours post injection (91). S-diclofenac perfusion (10 and 30 $\mu$M) protects against ischemia/reperfusion injury in isolated rat heart, which is mediated partly by opening KATP channels, and possibly by increasing cysteine uptake and GSH synthesis. In this model, S-doclofenac also reduces creatine kinase (CK) and lactate dehydrogenase (LDH) release and decreases the inhibitory effect of diclofenac on protective PGI$_2$ production (132). Intravenous injection of H$_2$S-releasing aspirin (ACS14; 0.1 mmol/kg) produces a slight transient rise in plasma H$_2$S (0.55 to 1.4 $\mu$mol/l that remains slightly elevated (0.62 $\mu$mol/l) at 150 minutes (145). Oral administration for 7 days of equimolar doses (~0.12 mmol/kg) of ACS14, ACS21 (a metabolite of ACS 14), or ADT-OH has no effect on systemic blood pressure, heart rate, but increases plasma GSH, and increases cardiac and aortic GSH. ACS14 also produces a concentration-dependent increase in HO-1 promoter activity in NIH3T3–HO-1-luc cells. ACS14 does not adversely affect aspirin’s ability to inhibit thromboxane synthesis (145). I.P injection of ACS14 or ADT-OH (both 0.1 mmol/kg) increases plasma H$_2$S from 0.4 to ~0.65 $\mu$M in 15
minutes with a 2\textsuperscript{nd} peak (0.5 μM) at 12-24 hours which is attributed to elevated plasma cysteine and subsequent metabolism to H\textsubscript{2}S. Both ACS14 and ADT-OH decrease plasma homocysteine and malonyldialdehyde (an indicator of oxidative stress) and increase total (reduced plus disulfide) cysteine and GSH. These drugs may have additional cardiovascular benefit by lowering plasma homocysteine [43].

H\textsubscript{2}S-donating sildenafil (ACS6) combines the inhibitory action of sildenafil on protein kinase G (PKG) with an H\textsubscript{2}S inhibition of protein kinase A (PKA) in porcine pulmonary arterial endothelial cells. Collectively, these inhibit superoxide formation and gp91\textsuperscript{phox} expression suggesting ACS6 may be effective in treating adult respiratory distress syndrome (ARDS). H\textsubscript{2}S release from 10 μM NaHS peaks in 30 min, whereas H\textsubscript{2}S release from 10 μM ACS6 peaks in 120 min. Furthermore, more H\textsubscript{2}S is released from ACS6 than NaHS and only 25% mole/mole H\textsubscript{2}S is released from either NaHS or ACS6. ACS6 release of H\textsubscript{2}S is ~4 times greater when incubated with endothelial cells than in buffer [111]. In rabbit corpus cavernosum ACS6 protects against oxidative stress by stimulating both PKA (H\textsubscript{2}S effect) and PKG (sildenafil effect). There is no specific vasodilatory benefit from H\textsubscript{2}S released by ACS6 [142], which seems to be more or less typical for all synthetic H\textsubscript{2}S donating drugs.

Sodium sulfide (NaHS) and sodium sulfide (Na\textsubscript{2}S) have long been used to generate H\textsubscript{2}S. While these are frequently called “H\textsubscript{2}S donors”, and have even been reported to slowly release H\textsubscript{2}S [111], they are sulfide salts and when placed in water their dissociation and subsequent H\textsubscript{2}S formation is nearly instantaneous [84]. Caution should be exercised with Na\textsubscript{2}S as this is a strong alkali. Na\textsubscript{2}S in sterile, buffered solution is produced by Ikaria as IK-1001 and is currently in clinical trials for reduction of reperfusion/injury (NCT00858936). Calcium sulfide CaS has
recently been shown to have similar actions (94), but does not appear to convey any distinct advantage over the other salts.

Recent reviews: 9, 20, 104, 128, 153, 154, 173, 205.

**Separating hype from hope**

There is little doubt that exogenous H$_2$S affects a myriad of physiological systems and many studies have been corroborated to some extent by compounds that can theoretically alter endogenous H$_2$S production. Moreover, the beneficial results obtained with the H$_2$S “donating” compounds is encouraging and bespeaks of wide-ranging potential. However, as is often the case with a novel and exciting field, critical details can be missed or overlooked in the accompanying exuberance. The following sections take a careful look at a number of areas that require a thoughtful and more thorough analysis.

**H$_2$S chemistry - what can H$_2$S do?**

Sen et al. 2010 (137) in their review state that “H$_2$S is a strong oxidant” and, although H$_2$S is more commonly thought of as a strong reducing agent, Kabil and Banerjee (66) show it is a relatively weak reducing agent, especially when compared to other intracellular thiols such as glutathione. H$_2$S has also been proposed to serve as a potent antioxidant, however, its very low concentration in tissues argues against this as well, and the protective effects cannot be completely accounted for by direct reactions with oxidants (21). Furthermore, because H$_2$S inhibits oxidative phosphorylation at ~20-40 μM (6, 24), and these concentrations greatly exceeded in experimental conditions involving either exogenous H$_2$S or H$_2$S donors, it can be difficult to separate specific H$_2$S effects from general metabolic depression or even more general reducing and/or antioxidant actions.
Blood and tissue H₂S concentrations - what is real and what is artifact?

The overwhelming majority of studies and reviews on the biology of H₂S refer to “physiological” concentrations of 20–40 μM H₂S in blood (with some reports of plasma H₂S approaching 300 μM). In turn, 20–300 μM H₂S has been used to validate many “physiological” experiments. However, reports of plasma H₂S >1 μM have been relatively recent, prior to the year 2000 most reported values were <1 μM (Fig. 5) and these early studies were largely ignored by those that followed. That plasma H₂S levels >10 μM are unrealistic has been argued based on both practical as well as methodological considerations (116). In addition, the use of newer methods, especially a polarographic (amperometric) sensor that directly measures H₂S gas fails to find H₂S approaching even 1 μM (186). There are also a number of other practical considerations, namely; 1) there is no odor of H₂S in plasma which there should be even at 1 μM, 2) H₂S rapidly equilibrates across the lung and would be readily exhaled if it existed in plasma (59, 169), 3) many of the reported plasma values would exceed toxic levels based on air quality standards (Table 1), 4) relative to point 2, it has been calculated that there is insufficient sulfur in the body to sustain H₂S production at this level (41), 5) H₂S is rapidly consumed by tissues in the presence of oxygen (Fig. 3; 82, 118, 138), 6) H₂S inhibits mitochondrial respiration at 20–40 μM. Furthermore, because H₂S is metabolized by the mitochondria as fast as it is generated under normoxic conditions, H₂S will increase only when tissue PO₂ falls.

In a recent study Wintner et al. (188) examined kinetics of plasma H₂S using a newly developed monobromodimane–based assay (MBA) and compared this to the polarographic sensor. Similar to the findings of Whitfield et al. (186), they observed that exogenous H₂S rapidly disappears from whole blood but disappears slowly when added to plasma or buffer. However,
when measured with the MBA, total sulfide increased and decayed slowly in blood, similar to that observed in plasma and buffer. Bolus injections or continuous infusion of H$_2$S exhibited similar discrepancies between the MBA and polarographic measurements, i.e. more sulfide is detected by the former. The authors propose that the MBA is measuring sulfide in a “reversible sulfide sink” which is most likely a persulfide. This they propose can be readily mobilized under physiological conditions and represents the true “biologically available sulfide in vivo”. There are several problems with this assumption. First, this persulfide cannot be in the plasma as the MBA and polarographic responses are the same and therefore it must reside in red blood cells. Second, the amount of sulfide injected or infused greatly exceeds the amount measured by the MBA; assuming plasma volume is 4% of body weight, bolus injection 4 mg/kg of sodium sulfide would theoretically increase plasma sulfide to 312 µM, yet the MBA only measured 4.5 µM sulfide, less than 2% of the injected dose. Infusion of sulfide produces similar results, after one hour of infusion of 20 mg/kg/h, blood sulfide measured with the MBA was 4.0 µM, whereas if the sulfide was confined to the plasma its theoretical concentration would be over 6,300 µM. Even if distributed throughout the entire body water, sulfide would still be in excess of 420 µM, again less than 1% of the infused dose. Third, the authors provide no evidence that this extra sulfide can in fact be readily mobilized and is therefore biologically available. Fourth, a simple experiment can test the hypothesis that exogenous sulfide can be released from a persulfide pool. In this experiment H$_2$S is measured with polarographic sensor and a strong reducing agent, dithiothreitol (DTT), known to liberate sulfane sulfur is added to blood before and after exogenous H$_2$S. As shown in Fig. 6, raising the concentration of dithiothreitol (DTT) in buffer to 10 mM increased current flow equivalent to approximately one-fifth of the response produced
by increasing H\textsubscript{2}S concentration to 50 \(\mu\text{M}\) (panel A). A similar 10 mM increase in DTT in whole blood produced an identical increase in current flow and the presence of DTT did not affect the response to a subsequent addition of H\textsubscript{2}S (panel B). When H\textsubscript{2}S was added to untreated blood (panel C), the H\textsubscript{2}S was rapidly consumed. Addition of DTT to the H\textsubscript{2}S-spiked blood produced the same response as DTT addition to buffer or untreated blood, i.e., there was no evidence of liberation of H\textsubscript{2}S from sulfane sulfur over the 10 minute treatment period. A second addition of H\textsubscript{2}S to the H\textsubscript{2}S-DTT-treated blood produced a response similar to that produced by addition of H\textsubscript{2}S to buffer or untreated blood. The consumption of H\textsubscript{2}S by untreated blood and the inability of DTT treated blood to consume H\textsubscript{2}S suggests that H\textsubscript{2}S is consumed (presumably oxidized) in blood during reduction of another blood constituent and that this is prevented by the high concentrations of another strong reductant, DTT.

Thus, one must question whether a reported fall in plasma H\textsubscript{2}S concentration from 41 to 18 \(\mu\text{mol/l}\) in CSE gene-deleted mice is really key evidence for H\textsubscript{2}S as a “physiologic” gasotransmitter of comparable importance to nitric oxide and carbon monoxide (42). Clearly, one must also question the numerous reports showing elevated (>10 \(\mu\text{M}\)) plasma H\textsubscript{2}S concentrations achieved with the H\textsubscript{2}S-donating drugs. Also worrisome are clinical trials that use these inaccurate methods to evaluate plasma hydrogen sulfide as a prognostic indicator of shock-related mortality (NTC 01088490).

Similar arguments can be made against excessive tissue H\textsubscript{2}S concentrations and tissue production. Recent studies have shown that H\textsubscript{2}S is consumed by tissues in the presence of oxygen and H\textsubscript{2}S production is only observed under hypoxic or anoxic conditions (41, 118). In fact, even exogenous H\textsubscript{2}S is quickly and efficiently consumed by tissues at oxygen partial
pressure (Po2) greater than 10 mmHg (118). By comparison, water or tissue samples in
equilibrium with room air typically have a Po2 greater than 140 mmHg. In addition,
unphysiologically high cysteine (usually 10 mM, compared to normal <1 mM) is used in tissue
production studies and serine and homocysteine are typically absent. This can artificially
increase the rate of H2S production and shuttle sulfur metabolism through normally minor
metabolic pathways (149). It is also important to stress that, to date no study has identified any
stimulus for H2S production in cells in real time and under physiological conditions, other than
showing an inverse relationship between H2S production and Po2.

Recent reviews: 24, 96, 116, 163.

Does H2S fulfill the criteria of a “gasotransmitter”?

In a recent review, Linden et al. (96) critically evaluated the criteria for a
“gasotransmitter” as originally proposed by Wang (179) and concluded that there are still
sufficient questions remaining before H2S can be accepted as a biologically relevant signaling
molecule. Foremost amongst these is the discrepancy between tissue and plasma concentrations
and the dose of exogenous H2S needed to produce physiological responses. In addition, there is
relatively little evidence regarding how H2S production or tissue concentrations are regulated.
Presumably, these issues will be resolved when the methodology improves, as historically been
the case with numerous other signaling molecules. There are other questions that must be
resolved. Is H2S only transiently present in blood and tissue and it does it serve to initiate
downstream effects that can be sustained for lengthy periods? Is H2S merely a byproduct or
intermediate of the “true” signaling molecule? Are there other more relevant biochemical
processes that are also inhibited by the promiscuity of “classical” inhibitors of H$_2$S biosynthesis that have led us astray? If H$_2$S is the principal signaling moiety, can H$_2$S-donating drugs be targeted for tissue-specific H$_2$S release? It would seem that these are pressing questions that if answered may save considerable time and effort as we attempt to target H$_2$S pathways to treat the variety of diseases that have been implicated in H$_2$S pathophysiology.

Recent review: 96.
H$_2$S has been associated with life from the onset, at times supporting it as a useful substrate and at times destroying it. It is not surprising that through evolution animals have learned to live in and around H$_2$S and have ultimately incorporated this versatile molecule into their biochemistry. Recent awareness of this fact has greatly expanded the field of “gasotransmitters”. However, unlike its predecessor, nitric oxide, whose discovery was met with initial skepticism, H$_2$S has been readily embraced by the scientific community and quickly targeted for its therapeutic potential. This exuberance has at times let enthusiasm reign over skepticism. As this field begins to mature, the need to recalibrate this balance is becoming increasingly evident.

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Figure 1. Potential pathways for H$_2$S production and metabolism. Inset shows potential H$_2$S production from carbonyl sulfide. Abbreviations: CA, carbonic anhydrase; CBS, cystathionine $\beta$-synthase; CDO, cysteine dioxygenase; CSD, cysteinesulfinate decarboxylase; CSE, cystathionine $\gamma$-ligase; MST, 3-mercaptopyruvate sulfurtransferase. From: 65, 66, 143,149.

Figure 2. Mitochondrial oxidation of H$_2$S. Two sulfide:quinone oxidoreductase (SQR) in the mitochondrial membrane (stippled box) oxidize sulfide to the level of elemental sulfur, simultaneously reducing a cysteine disulfide, and resulting in formation of a persulfide group at one of the SQR cysteines (SQR-SSH). Sulfur dioxygenase (SDO) then oxidizes one persulfide to sulfite (H$_2$SO$_3$), consuming molecular oxygen and water in the process. The second persulfide is transferred from the SQR to sulfite by sulfur transferase (ST) producing thiosulfate (H$_2$S$_2$O$_3$). Electrons from H$_2$S are fed into the respiratory chain via the quinone pool (Q), and ultimately transferred to oxygen by cytochrome $c$-oxidase (complex IV).

Figure 3. Inverse relationship between H$_2$S and O$_2$ in tissues. A) H$_2$S production by homogenized rat lung is converted to net H$_2$S consumption in the presence of O$_2$. Tissue was de-oxygenated by gassing with 100% nitrogen and primed with cysteine (1 mM) and $\alpha$-ketoglutarate(1 mM) and placed in a sealed container. H$_2$S and O$_2$ were continuously recorded with amperometric electrodes. H$_2$S is expressed as total sulfide (H$_2$S plus HS$^-$) calculated from tissue pH. As O$_2$ falls H$_2$S concentration increases; injection of a small air bubble (arrow) immediately decreases H$_2$S concentration which then resumes after the O$_2$ has been consumed.
B) effect of O$_2$ on H$_2$S consumption by pulmonary arterial smooth muscle cells (PASMC), homogenized bovine lung (lung) and purified mitochondria (mito) compared to O$_2$ dependence of hypoxic pulmonary vasoconstriction of isolated bovine pulmonary arteries (vessel). Percent activity refers to the degree of H$_2$S consumption (100% = all H$_2$S consumed) or percent of hypoxic contraction (100% = maximum vessel contraction). Half maximal H$_2$S consumption and vessel contraction occurs at approximately the same PO$_2$. A) Modified from 118; B) modified from 138.

**Figure 4.** H$_2$S “donating” compounds. A. Diallyl trisulfide, one of two active components of garlic from which H$_2$S is synthesized. B. Sulforaphane, the sulfur-containing moiety in broccoli. C. H$_2$S releasing compound GYY4137. D. Lawesson’s reagent. E. S-propargyl cysteine, a cysteine analog. F. Anethole trithione hydroxide, ADT-OH the dilthiolethione sulfur donor frequently added to a variety of compounds such as diclofenac (G) or valproate (H). I, J. methanethiosulfonate derivatives of valproate. K-M. Other H$_2$S donors that have been combined with L-DOPA. ATB, Antibe Threapeutic (Hamilton, Canada); ACS, CTG- Pharma, (Milan Italy).

**Figure 5.** Frequency distribution of papers reporting plasma H$_2$S at various concentrations. Prior to 2000 nearly all studies reported H$_2$S as undetectable (<1). Subsequently the number of studies reporting H$_2$S between 10 and 100 μM has progressively increased (most between 20 and 40 μM) with a few studies reporting values in excess of 100 μM (most between 200 and 300 μM). Modified from Olson 116.
Figure 6. Lack of evidence of sulfane sulfur in trout blood. A. Dithiothreitol added to Cortland buffer produces a characteristic current in the polarographic H$_2$S sensor. Addition of H$_2$S increases current flow five-fold. B. Similar additions of DTT and H$_2$S to heparanized trout blood produce identical responses. C. H$_2$S added to blood without prior DTT treatment rapidly disappears. Addition of DTT to H$_2$S-treated blood produces the same response as DTT addition to Cortland (A) or untreated blood (B) and subsequent addition of H$_2$S in the presence of DTT also produces the same response as H$_2$S in panels A and B. Each addition of DTT will produce a final DTT concentration of 10 mM and each addition of H$_2$S will increase the final concentration by 50 µM (Whitfield, Kreimier and Olson, unpublished).
<table>
<thead>
<tr>
<th>Ambient H₂S (ppm)</th>
<th>Equivalent total plasma sulfide (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01-0.3</td>
<td>0.003-0.1</td>
<td>Threshold for detection</td>
</tr>
<tr>
<td>1-3</td>
<td>0.3-1</td>
<td>offensive odor, headaches</td>
</tr>
<tr>
<td>10</td>
<td>3.3</td>
<td>8-hour occupational exposure limit in Alberta, Canada</td>
</tr>
<tr>
<td>15</td>
<td>4.9</td>
<td>15 minute exposure limit in Alberta, Canada</td>
</tr>
<tr>
<td>20-50</td>
<td>6.5-16.2</td>
<td>eye and lung irritation</td>
</tr>
<tr>
<td>100</td>
<td>32.5</td>
<td>olfactory paralysis</td>
</tr>
<tr>
<td>250-500</td>
<td>81.1-162.3</td>
<td>pulmonary edema</td>
</tr>
<tr>
<td>500</td>
<td>162.3</td>
<td>sudden unconsciousness (&quot;knockdown&quot;), death within 4- to 8- hours</td>
</tr>
<tr>
<td>1000</td>
<td>324.5</td>
<td>immediate collapse, breathing ceases within several breaths</td>
</tr>
</tbody>
</table>
All except Equivalent total plasma sulfide column modified from Guidotti (46).

a. Equivalent plasma sulfide calculated after Whitfield et al. (186, supplemental information) assuming \( \text{H}_2\text{S} \) equilibrates across the alveolar membranes (169), Henry's Law constant for \( \text{H}_2\text{S} \) at 37°C and 140mM NaCl is 0.0649 M·atm\(^{-1} \) (27), and 20% of total sulfide exists as \( \text{H}_2\text{S} \) gas (115).
Fig.1

L-Methionine → Homocysteine → Cystathionine → Cysteine → Cystathionine + H₂S → Lanthionine + H₂S → CBS → Cysteine → Cysteinesulfinate → Hypotaurine

Homocysteine → CBS → Homolanthionine + H₂S

L-Serine + H₂S → L-Cysteate + H₂S

Pyruvate + NH₃ + H₂S → Carbonyl Sulfide

Carbonyl Sulfide → CSE → Cysteine thioether + H₂S → 3-Mercaptopyruvate + H₂S

3-MST → Thiosulfate cycle ↔ GSSG + SO₃²⁻ + H₂S
Fig. 2

\[ 2\text{H}_2\text{S} \rightarrow \text{H}_2\text{SO}_3 \rightarrow \text{H}_2\text{S}_2\text{O}_3 \]

\[ \text{SQR} \]

Matrix

Cytosol

\[ 2\text{e}^- \rightarrow \text{Q} \rightarrow \text{III} \rightarrow \text{IV} \]

\[ \frac{1}{2}\text{O}_2 \rightarrow 2\text{H}_2\text{O} \]
Fig. 3

A

\[ \mu M \text{ sulfide or } O_2 \]

\[ \text{Time (min)} \]

B

\[ \% \text{ Activity} \]

\[ \text{PO}_2 \text{ (mmHg)} \]

mito → cyto → tiss

lung

PASMC

vascul
Fig. 4

A: diallyl trisulfide

B: $\text{H}_3\text{C-S-CH}_2\text{N}=\text{C}=\text{S}$

sulfuraphane

C: $\text{MeO-}[\text{P-S}^+\text{H}_2\text{N-O}]$

GYY4137

D: Lawesson’s reagent

E: $\text{CH}_2\text{S-S-CH}_2\text{OH}$

S-propargyl cysteine

F: $\text{HO-}[\text{CH}_2\text{S-S-S-}]$

ATD-OH

G: $\text{CH}_2\text{C}=\text{O}[\text{S-CH}_2\text{S-S-S-}]$

S-diclofenac; ATB-337, ACS 15

H: $\text{HO-}[\text{CH-S-S-S]}$

ACS 2

I: $\text{HO-}[\text{CH-S-S-CH}_3]$ ACS 33

J: $\text{HO-}[\text{CH-S-S-CH}_3]$ ACS 43

K: $\text{HO-}[\text{CH-S-S-}]$ ACS 5

L: $\text{HO-}[\text{CH-S-S-S-}]$

ACS 50

M: $\text{HO-}[\text{CH-S-S-S-}]$

ACS 81
Fig 5

Number of Papers

<2000  00-01  02-03  04-05  06-07  08-09  2010

Plasma H2S (µM)

- <1
- 1-10
- 10-100
- >100
Fig 6