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The therapeutic potential of hydrogen sulfide: separating hype from hope

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Olson KR. The therapeutic potential of hydrogen sulfide: separating hype from hope. *Am J Physiol Regul Integr Comp Physiol* 301: R297–R312, 2011. First published May 4, 2011; doi:10.1152/ajpregu.00045.2011.—Hydrogen sulfide (H_2S) 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 proinflammatory and anti-inflammatory actions and proapoptotic 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 H_2S-"donating" compounds is understandable and a number of these function-targeted drugs have been developed and show clinical promise. However, the concentration of H_2S in tissues and blood, as well as the intrinsic factors that affect these levels, has not been resolved, and it is imperative to address these points to distinguish between the physiological, pharmacological, and toxicological effects of this molecule. This review will provide an overview of H_2S metabolism, a summary of many of its reported "physiological" actions, and it will discuss the recent development of a number of H_2S-donating drugs that show clinical potential. It will also examine some of the misconceptions of H_2S chemistry that have appeared in the literature and attempt to realign the definition of "physiological" H_2S concentrations upon which much of this exuberance has been established.

hydrogen sulfide-donating drugs; vasoactivity; ischemia reperfusion injury; sulfur cycle; gasotransmitter

THE INITIAL DISCOVERY by Hideo Kimura's group that hydrogen sulfide (H_2S)[1] was a biologically relevant signaling molecule (reviewed in Ref. 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_2S 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 reheightened interest in the dietary sources of H_2S, and it has spawned the development of a number of H_2S-"donating" drugs, many of which are in various stages of clinical trials. However, it is becoming increasingly evident that there is still much to be learned about the basic properties of H_2S measurement, metabolism, and signaling mechanisms. This review will provide an overview of the effects of H_2S on physiological systems, summarize the new H_2S-donating drugs that are showing clinical potential, and take a critical look at the some of the remaining uncertainties surrounding H_2S chemistry and tissue concentrations.

Hydrogen Sulfide as a Toxic Gas

The toxic effects of H_2S 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_2S has even gained notoriety in a recent spate of 220 suicide cases in less than 3 mo in Japan (107). Less is known of the effects of low-level ambient H_2S that are often associated with sewage plants, waste lagoons, natural gas/oil wells, and oil refineries, as well as a variety of other industrial applications. Recent studies on residents of Southeastern New Mexico exposed to these environments have shown positive correlations with H_2S exposure and impaired neurobehavioral functions compared with controls (73). This suggests that even "therapeutic" use of H_2S is not without potential hazards. Thresholds for the major effects of H_2S exposure are shown in Table 1.

The inhibitory effects of H_2S on mitochondrial cytochrome-*c* oxidase have been well characterized and this is generally assumed to be the focus of H_2S toxicity (34). However, the clinical presentation of poisoning by H_2S 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 H_2S 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 H_2S toxicity is closely correlated with H_2S concentration and considerably less dependent upon the duration of exposure. This suggests that animals can

[1] Unless otherwise noted, H_2S refers to the sum of dissolved H_2S gas and HS^-, often referred to as "sulfide". At physiological pH, S_2^- is assumed to be negligible.

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Table 1. *The effects of H_2S exposure*

Ambient H_2S, ppm	Equivalent Total Plasma Sulfide, μM[a]	Effects
0.01–0.3	0.003–0.1	Threshold for detection
1–3	0.3–1	offensive odor, headaches
10	3.3	8-h occupational exposure limit in Alberta, Canada
15	4.9	15-min exposure limit in Alberta, Canada
20–50	6.5–16.2	eye and lung irritation
100	32.5	olfactory paralysis
250–500	81.1–162.3	pulmonary edema
500	162.3	sudden unconsciousness ("knockdown"), death within 4 to 8 h
1000	324.5	immediate collapse, breathing ceases within several breaths

All except "Equivalent Total Plasma Sulfide" column modified from Guidotti (46). [a]Equivalent plasma sulfide calculated after Whitfield et al. (186, supplemental information), assuming H_2S equilibrates across the alveolar membranes (169), Henry's Law constant for H_2S at 37°C, 140 mM NaCl is 0.0649 $M \cdot atm^{-1}$ (27), and 20% of total sulfide exists as H_2S gas (115).

rapidly metabolize H_2S up to a critical level and, as a corollary, this efficient metabolic capacity should keep free H_2S at very low levels. These studies should, but have not often, raised questions regarding "physiological" concentrations of H_2S 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 H_2S, 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 H_2S 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 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 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 regulator, if not the primary one, of cysteine availability in vivo. By oxidizing excess and presumably toxic cysteine, CDO provides a constant and low-level background of cysteine for H_2S and sulfane sulfur biosynthesis. This may be important in preventing excessive H_2S production (33).

H_2S can be produced from cysteine via a variety of biochemical pathways. Early studies indicated that cystathionine β-synthase (CBS) was the predominant enzymatic pathway for H_2S production in the brain, whereas cystathionine γ-lyase (CSE, also known as CGL) was responsible for H_2S production in the

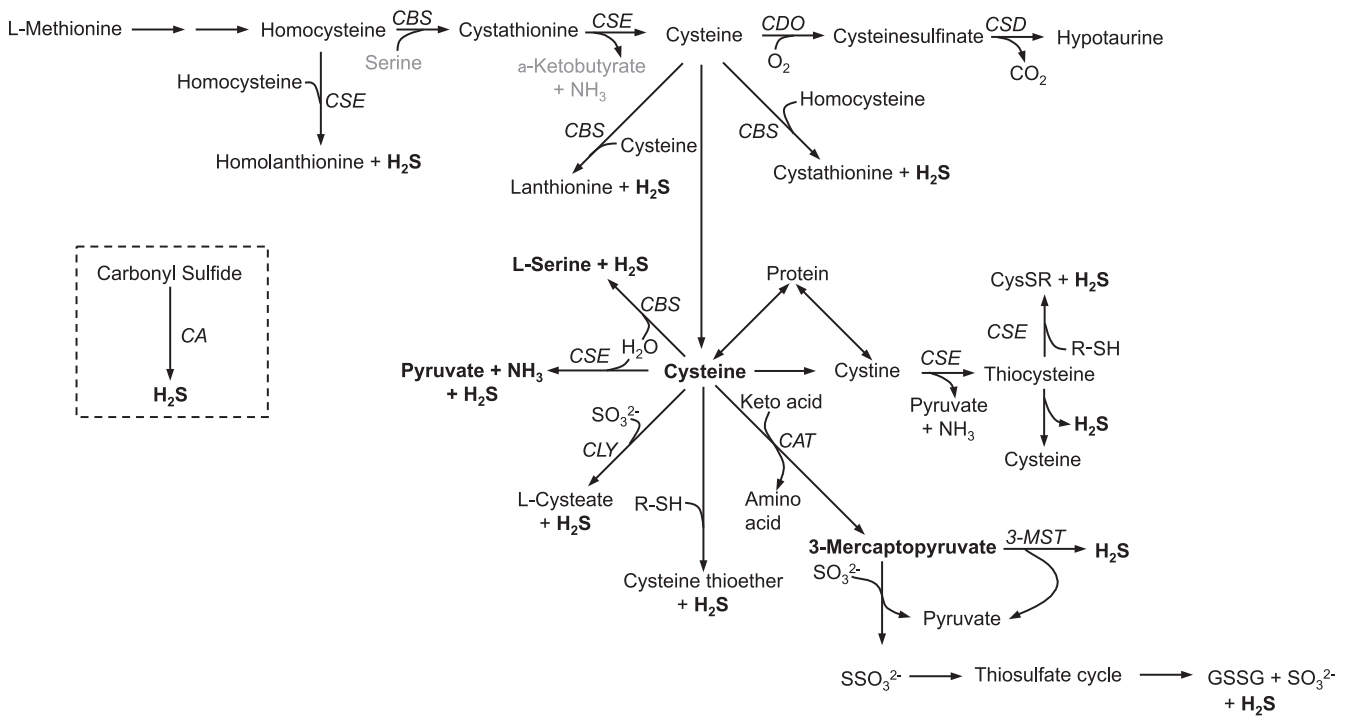

Fig. 1. Potential pathways for H_2S production and metabolism. Inset shows potential H_2S production from carbonyl sulfide. CA, carbonic anhydrase; CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CDO, cysteine dioxygenase; CLY, cysteine lyase; CSD, cysteine sulfinate decarboxylase; CSE, cystathionine γ-ligase; MST, 3-mercaptopyruvate sulfurtransferase; R-SH, thiol. [Modified from Julian et al. (65), Kabil et al. (66), Singh et al. (143), and Stipanuk and Ueki (149).]

vasculature (75). Recent studies have shown that 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 α-ketoglutarate, resulting in 3 mercaptopyruvate, which is then desulfurated by MST. In addition to H_2S, 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 H_2S production in the brain, but they also suggested that the H_2S is immediately "stored" as sulfane sulfur, the latter serving as a less labile form of H_2S 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 H_2S and because these conditions may not be routinely encountered intracellularly, the significance of the CAT/MST pathway in H_2S synthesis remains questionable. Both CBS and CSE have recently been shown to circulate in human plasma and to generate H_2S from cysteine or homocysteine plus cysteine (13). This generation of H_2S has been proposed not only to reduce circulating homocysteine, but also to protect the endothelium from oxidative stress (12).

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 that, 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 NO nor calmodulin appears 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*) H_2S generation from cysteine is primarily catalyzed by CSE, *2*) H_2S 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_2S generated under resting conditions, *3*) H_2S biosynthesis can occur independent of cysteine; condensation of two molecules of homocysteine, catalyzed by CSE, yields homolanthionine and H_2S, and may account for as much as 30% of the total H_2S 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_2S and thereby contribute to the cardiovascular pathology associated with this condition (23).

Commonly used inhibitors of CSE include propargyl glycine (PPG) and β-cyanoalanine. Aminooxyacetate (AOA) is commonly used to inhibit CBS and hydroxylamine 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_2S production; furthermore, they are often poorly absorbed by tissues (153).

Other Potential Biosynthetic Pathways

There are numerous other potential metabolic pathways for H_2S generation that have been described in invertebrates (Fig. 1; Ref. 65), but these have not been systematically evaluated in mammalian tissues. The resurgent interest in H_2S 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_2S can also be affected by a variety of other sulfur-donating molecules. One potentially novel pathway that needs to be investigated is H_2S production from carbonyl sulfide (COS; chemical structure: O=C=S). Like H_2S, 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 ACh or the calcium ionophore, A23187 (7). In solution, COS slowly decomposes to H_2S, 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_2S production and subsequent signaling events remains to be determined.

Metabolism (Inactivation)

Oxidation of H_2S 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

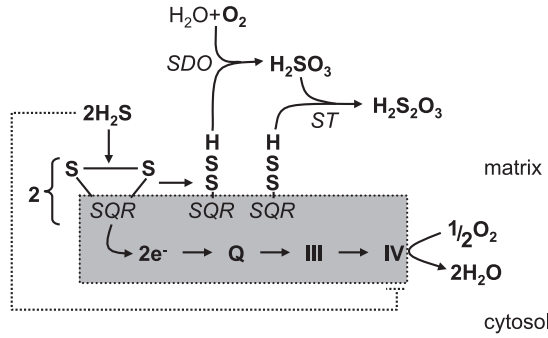

Fig. 2. Mitochondrial oxidation of H_2S. Two sulfide:quinone oxidoreductase (SQR) in the mitochondrial membrane (stippled box) oxidize sulfide to the level of elemental sulfur, simultaneously reducing a cysteine disulfide, and resulting in the formation of a persulfide group at one of the SQR cysteines (SQR-SSH). Sulfur dioxygenase (SDO) then oxidizes one persulfide to sulfite (H_2SO_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 ($H_2S_2O_3$). Electrons from H_2S are fed into the respiratory chain via the quinone pool (Q), and ultimately transferred to oxygen by cytochrome-*c* oxidase (complex IV).

formation of persulfide groups at one of the SQR cysteines. Sulfur dioxygenase (SDO) then oxidizes one of the persulfides to sulfite (H_2SO_3), consuming molecular oxygen and water in the process. Sulfur from the second persulfide is transferred from the SQR to sulfite by sulfur transferase producing thiosulfate ($H_2S_2O_3$). Most thiosulfate is further metabolized to sulfate by thiosulfate reductase and sulfite oxidase. Electrons from H_2S 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_2S metabolism, and 1 mol of oxygen is consumed for every mol of H_2S oxidized along the electron transport chain (53). Oxidation of H_2S to thiosulfate requires additional oxygen at the level of SDO, resulting in a net utilization of 1.5 mol of oxygen per mol of H_2S (or 0.75 mol of O_2 per mol H_2S; Ref. 82). Metabolism of H_2S 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_2S concentrations are very low. Interestingly, the statin, atorvastatin, increases H_2S production in perivascular adipose tissue by producing coenzyme Q_9 deficiency and thereby inhibiting mitochondrial oxidation (189).

The relationship between H_2S and O_2 consumption is classical hormesis; at low concentrations, H_2S stimulates oxygen consumption (and may even result in net ATP production), whereas it is inhibited by elevated H_2S. This was originally shown in invertebrates and lower vertebrates and more recently demonstrated in the mammalian colon (45). At higher concentrations, H_2S inhibits the respiratory chain by directly inhibiting cytochrome-*c* oxidase (24). The exact H_2S concentration at which this occurs is unclear; purified cytochrome-*c* oxidase is inhibited by <1 μM H_2S, whereas progressively greater (2 or 3 orders of magnitude) higher H_2S 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_2S and may not be fully inhibited until H_2S concentrations reach 40–50 μM (6, 24). This may reflect diffusion limitation as the enzyme becomes further removed from the exogenously administered H_2S. It also should provide a cautionary note in interpreting studies that routinely employ 100 μM–1 mM H_2S to demonstrate a "physiological" effect. The converse, i.e., the effect of O_2 on H_2S consumption, is discussed in *H_2S and oxygen sensing*.

H_2S Biology

Interest in H_2S 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 H_2S on a particular system were unknown (87, 196). The following sections provide a brief overview of H_2S biology. For further details, the reader is referred to a few of the most recent reviews following each section.

H_2S and the nervous system. Potentiation of the *N*-methyl-D-aspartate (NMDA) receptor with the resultant alteration of long-term potentiation (LTP) in the hippocampus was the first biological effect ascribed to H_2S (1). Not long thereafter, it was noted that patients with Down syndrome had elevated concentrations of H_2S in cerebral spinal fluid. This would be predicted from the fact that chromosome 21 encodes CBS (which may be the major H_2S-producing enzyme in the brain) and is overexpressed in these patients (70). It has also been suggested that deficiencies in H_2S biosynthesis are associated with Alzheimer's disease (see Ref. 37, reviewed in Ref. 130) and that exogenous H_2S may have therapeutic potential by reducing amyloid beta protein plaques (201). H_2S 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 H_2S have been demonstrated in a number of neurological systems. H_2S 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, H_2S has been shown to mediate cerebral ischemic damage (129) and produce vanilloid receptor 1-mediated neurogenic inflammation in airways (170).

H_2S increases cAMP production in neurons and subsequent activation of PKA may account portion of the LTP. Other functions of H_2S include upregulation of GABA B receptor and neuronal hyperpolarization via K_{ATP} 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. For recent reviews, see Refs. 56, 130, 160 and 144.

H_2S and the gastrointestinal system. The initial interest in H_2S in the gastrointestinal (GI) system stemmed from the well-known production of H_2S 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 H_2S in the colon than any other segment of the GI tract; however, anti-inflammatory actions of H_2S in the stomach appear to be of important therapeutic value and other areas have received increased attention as well.

H_2S 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 muscularis mucosa, cell mucosa, and lamina propria but not associated with goblet, crypt, and epithelial cells (105).

H_2S relaxes smooth muscle in the stomach (28) intestine (113), and colon (29). The mechanisms of H_2S on GI 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 H_2S acts partly via activation of myosin light-chain phosphatase (28); in the colon, the effects of H_2S are independent of intracellular calcium and not mediated through known K^+ channels, myosin light-chain phosphatase, or Rho kinase (29), and in the ileum, H_2S relaxation is independent of extrinsic or enteric nerves, NO, K_{ATP}, and KCa^+ channels (113). H_2S 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 H_2S, which acts via phosphorylation of AKT protein kinase (57).

H_2S stimulates chloride secretion in the intestine by targeting vanilloid receptors (transient receptor potential vanilloid 1) on afferent nerves, which, in turn, activate cholinergic secretomotor neurons via release of substance P (79).

H_2S has both anti-inflammatory and inflammatory effects in the GI tract; however, the former is perhaps better characterized and appears to be of therapeutic value. In the colon, H_2S is anti-inflammatory and enhances ulcer healing, independent of nitric oxide synthase and K_{ATP} channel involvement (176). H_2S production is increased in experimental models of colitis and H_2S protects against and promotes resolution of this colitis (177). However, H_2S 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, H_2S is a mediator of inflammatory caerulein-induced pancreatitis (17, 158, 159). H_2S acts through ICAM-1 expression and stimulates neutrophil adhesion through the NF-κB and Src-family kinases (157). However, H_2S 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 H_2S inhibits it (39). Bile acids increase liver CSE expression via activation of the farnesoid X receptor, the resultant H_2S production is proposed to maintain vasodilation and minimize the chance for portal hypertension (131). For recent reviews, see Refs. 64, 71, 96, 106, 133, and 175.

H_2S and the cardiovascular system. Collectively, the involvement of H_2S on heart and blood vessel physiology has received more attention than any other organ system, even though the therapeutic applications of H_2S are less evident.

The vasodilatory effects of H_2S 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). H_2S-induced relaxation appears to depend on extracellular Ca^{2+} (203), and although K_{ATP} channels, are frequently assumed to mediate the H_2S 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, K_{ATP} channels are not involved at all in the response. H_2S 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_2S does not depend on vascular prostaglandin synthesis, PKC, or cAMP, nor does it involve superoxide or H_2O_2 production (77, 78, 204). Observations that H_2S sulfhydrates and may regulate biological activity of numerous proteins, including actin (109), suggests that additional key steps in H_2S-mediated vascular signaling are soon to be unraveled. However, even this mechanism has been questioned on the basis of the seemingly nonselectivity 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_2S may also indirectly relax blood vessels in vivo through its ability to inhibit angiotensin-converting enzyme and thus prevent formation of the vasoconstrictor ANG II.

Recent evidence has turned to H_2S as the elusive endothelium-derived hyperpolarization factor, the third endothelium-derived relaxing factor that, along with NO and prostacyclin, signals vasodilation (180). Crosstalk between H_2S, NO, and CO has been suggested to contribute to vasoactivity and, although CO inhibits CBS (8), interactions between H_2S and NO are far from resolved. NO production has been shown to be directly inhibited by H_2S (81), or indirectly stimulated by it through activating NF-κB, which activates the ERK1/2, which, in turn, activates inducible nitric oxide synthase (iNOS) (62). H_2S 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 H_2S production (8). There is also evidence that H_2S and NO may form a simple S-nitrosothiol with vasoactive properties of its own (184).

Reports of H_2S-mediated vasoconstrictory responses in mammalian systemic vessels are less common, and many of these show an endothelium-dependent effect that has been attributed to H_2S inactivation of NO. Low concentrations of H_2S (<200 μM) produce endothelium-dependent contraction of human internal mammary arteries and rat and mouse aortas (2, 81, 181), and low-dose H_2S infusion increases blood pressure in the rat (2). These contractions have been proposed to result from H_2S inactivation of endothelial NO via production of an inactive nitrosothiol (2, 181), whereas Kubu et al. (81) showed that H_2S directly inhibited NO production. Other studies suggest that H_2S may have direct, albeit modest, constrictory effects on systemic vascular smooth muscle. Lim et al. (95) observed 1 μM H_2S contractions of rat aortas that were partially independent of both the endothelium and K_{ATP} channels and due, in part, to down-regulation of cAMP. Direct H_2S-mediated vasoconstriction has been demonstrated in systemic vessels of nonmammalian vertebrates, and H_2S contracts pulmonary vessels in terrestrial mammals in response to hypoxia (32, 117, 118).

H_2S 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. H_2S has been shown to be both proinflammatory and anti-inflammatory, to reduce leukocyte adhesion, to inhibit platelet aggregation, and although it is proangiogenic, to reduce deleterious vascular remodeling that often accompanies vascular damage (35, 89, 155). H_2S is not only a mild antioxidant, but it also stimulates cysteine uptake and synthesis of glutathione. H_2S has been implicated in hypotension associated with septic and hypovolemic shock, and inappropriate H_2S regulation of insulin secretion in type II diabetes may contribute to macrovascular and microvascular pathologies (85). Inhibition of plasma renin activity by H_2S is antihypertensive in renin-dependent hypertensive rats (99) and can potentially augment the depressor effect of H_2S vasodilation.

While H_2S 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 H_2S or H_2S donors can mimic hypoxic preconditioning and postconditioning and that increased endogenous H_2S biosynthesis can also protect the heart from ischemia/reperfusion injury (reviewed by Refs. 35, 83, 156). Furthermore, the potential for H_2S-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 H_2S production (192), liver and small intestine (52, 198), skeletal muscle (49, 51), and cellular components of cutaneous tissue (50). As in the vasculature, H_2S has been proposed to combine with NO to produce a nitrosothiol with inotropic properties (195, 194). For recent reviews, see Refs. 11, 15, 36, 83, 114, 155, 156, 172, 180, and 207.

H_2S and the respiratory system. Much of the focus of H_2S 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 H_2S production (140). During chronic hypoxia and the associated pulmonary hypertension, plasma and lung tissue production of H_2S 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_2S reduces pulmonary arterial pressure (182, 199) but (surprisingly), this does not affect aortic pressure (199). Exogenous H_2S also decreases tissue GSSG and increases total antioxidant capacity (182). H_2S 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_2S also relaxes precontracted mouse bronchial smooth muscle via a mechanism that is independent of K_{ATP} channels, soluble guanylyl cyclase, cyclooxygenases 1 and 2, and tachykinins (80).

Plasma H_2S is reported to decrease in rats with oelic acid-induced lung injury; exogenous H_2S increases arterial Pa_{O_2}, decreases pulmonary edema and infiltration of polymorphonuclear cells, decreases IL-6 and IL-8, but increases IL-10, suggesting that endogenous H_2S production is decreased in this model of lung injury (93). CSE expression in airway and vascular smooth muscle decreases in ovalbumin-induced lung asthma, whereas exogenous H_2S 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_2S (3). These studies suggest H_2S is anti-inflammatory and anti-remodeling in a variety of lung pathologies in addition to hypoxia. For a recent review, see Ref. 120.

H_2S and the kidney. H_2S affects both the renal tubule and vasculature. H_2S is produced in the kidney by combined actions of CBS and CSE (191). Simultaneous administration of AOA and PPG (but neither independently) decreases glomerular filtration rate (GFR) and sodium and potassium excretion, whereas these are increased by infusion of either H_2S or Cys (191). In the two-kidney, one-clip rat model of renal vascular hypertension, exogenous H_2S decreases blood pressure, decreases plasma renin activity and ANG 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, H_2S production is down-regulated, GFR decreases, glomerular inflammation increases, and these effects that can be reversed by exogenous H_2S (138). In a mouse model of diabetic nephropathy induced by streptozotocin, plasma and renal cortex H_2S decrease, TGF-β1 and collagen IV increase, and these changes are prevented by exogenous H_2S (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 H_2S (197). In human patients, hemodialysis appears to lower plasma H_2S (125). For a recent review, see Ref. 14.

H_2S 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 H_2S in the corpus cavernosum and the potential for H_2S therapy in erectile dysfunction (26, 146). Human and rat vas deferens smooth muscle contains both CBS and CSE and is relaxed by H_2S (88). H_2S 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). H_2S also produces dilation in vaginal and clitoral cavernosal muscle strips in the rabbit (147). However, the role of H_2S in reproduction per se is unknown.

H_2S 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 H_2S to reduce methemoglobin, the estimation that acid-labile H_2S could be released from a variety of cytochromes, and the inhibitory effect of H_2S on cytochrome-*c* oxidase, it was only natural to assume that H_2S 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 H_2S concentrations (3:1, H_2S: 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 it is doubtful that H_2S concentrations even approach these levels in vivo. While H_2S concentrations may increase during toxic exposure, the adverse effect of sulfhemoglobin formation will be somewhat offset by a concomitant H_2S-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 H_2S (reviewed in Ref. 127). H_2S has recently been shown to form sulfheme 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. For a recent review, see Ref. 127.

H_2S and oxygen sensing. We initially proposed that H_2S 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_2S in the presence of oxygen (Fig. 3*A*) and observations that the rate of H_2S metabolism is coupled to tissue or mitochondrial oxygen at physiologically relevant P_{O_2}s (Fig. 3*B*). The relationship between oxygen consumption and H_2S production are considered in more detail below. Mechanisms of H_2S-mediated vasodilation were described previously

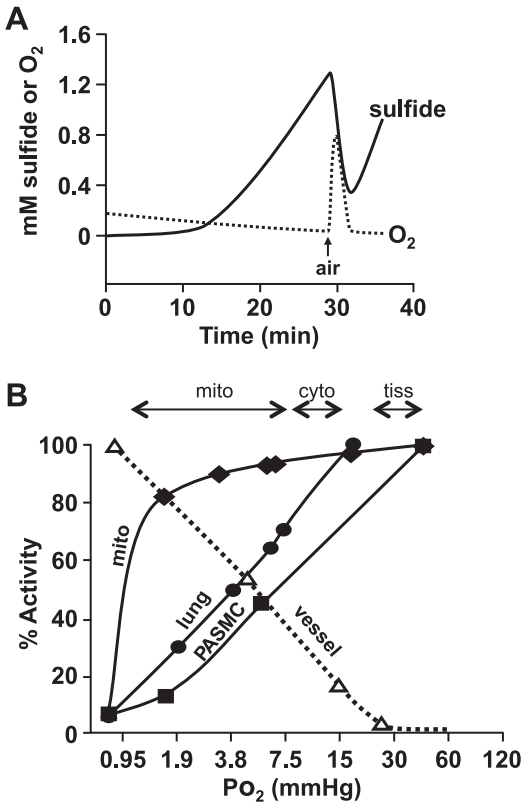

Fig. 3. Inverse relationship between H_2S and O_2 in tissues. *A*: H_2S production by homogenized rat lung is converted to net H_2S consumption in the presence of O_2. Tissue was deoxygenated by gassing with 100% nitrogen and primed with cysteine (1 mM) and α-ketoglutarate (1 mM) and placed in a sealed container. H_2S and O_2 were continuously recorded with amperometric electrodes. H_2S is expressed as total sulfide (H_2S plus HS^-) calculated from tissue pH. As O_2 falls, H_2S concentration increases; injection of a small air bubble (arrow) immediately decreases H_2S concentration, which then resumes after the O_2 has been consumed. *B*: effect of O_2 on H_2S consumption by pulmonary arterial smooth muscle cells (PASMC), homogenized bovine lung (lung), and purified mitochondria (mito) compared with O_2 dependence of hypoxic pulmonary vasoconstriction of isolated bovine pulmonary arteries (vessel). Percent activity refers to the degree of H_2S consumption (100% = all H_2S consumed) or the percentage of hypoxic contraction (100% = maximum vessel contraction). Half-maximal H_2S consumption and vessel contraction occurs at approximately the same P_{O_2}. [*A*: modified from Olson and Whitfield (118); *B*: modified from Olson et al. (119).]

in *H_2S and the cardiovascular system.* The mechanism of H_2S-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_2S 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_2S also is the initial stimulus in preconditioning and postconditioning effects associated with reperfusion injury and may also contribute to the pathology of reperfusion injury in unconditioned tissues. For recent reviews, see 14, 116, and 118.

H_2S and metabolism. Recent studies showing that inhaled H_2S can induce a "suspended animation-like state" in small mammals have heightened expectations of using H_2S 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_2S remain unclear. Baumgart et al. (12) showed in small rodents that while H_2S inhalation during hypothermia did not alter the hemodynamic and cardiac effects of hypothermia itself, it did improve mitochondrial respiration, and they proposed that this may be the benefit of exogenous H_2S during hypothermia. Interestingly, the authors also found that the H_2S 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)*. Clearly, more needs to be learned regarding the transition from mitochondrial oxidation of H_2S as an energy source to the inhibitory effects of H_2S on oxidative phosphorylation. For a recent review, see Ref. 4; for other general reviews, see Refs. 42, 48, 58, 67, 74, 75, 89, 90, 101, 110, 121, 136, 185, and 200.

Disorders of H_2S Metabolism

Relatively few clinical conditions are currently attributable to H_2S 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 H_2S oxidation, resulting in elevated tissue H_2S and associated toxicity. Paradoxically, however, $H_2S_3O_2$ 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 and is secondarily associated with a wide range of diseases, including diabetes insipidus, Down syndrome, neuoroblastoma, hepatoblastoma, and celiac disease; however, it 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). For reviews, see Refs. 66, 103, 137, and 187.

H_2S Donating Drugs

An appreciation, if not understanding, of the health benefits of sulfur springs, garlic, and cruciferous vegetables has been known since ancient times. Recent evidence suggests that the common denominator in these folk remedies may be their ability to produce H_2S. Considerable effort is now under way 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_2S-donating compound is attached to another drug. A few of the more common H_2S-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 accessed from the website "www.clinicaltrials.gov". A list of patent applications on H_2S-releasing molecules and dosages can be found in Bannenberg and Vieira (9).

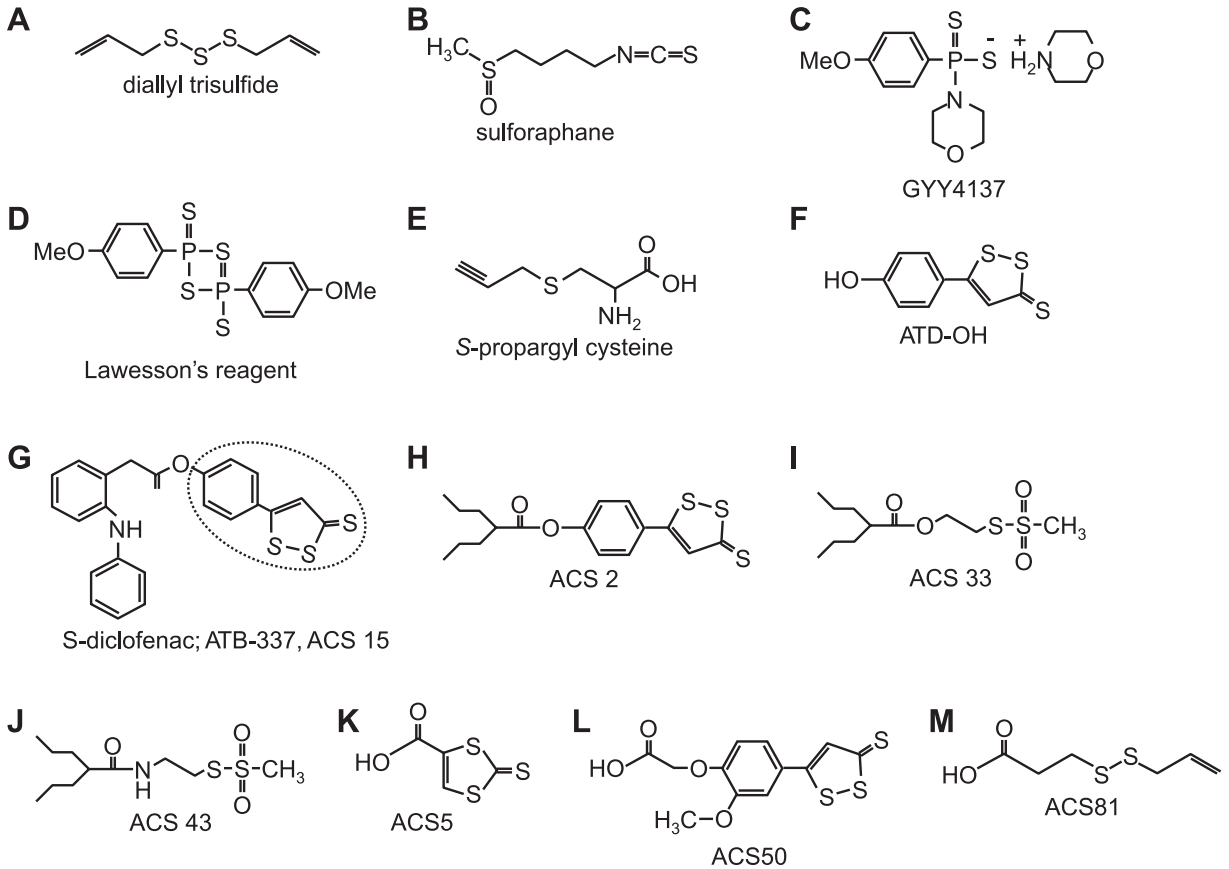

Fig. 4. H_2S-donating compounds. *A*: diallyl trisulfide, one of two active components of garlic from which H_2S is synthesized. *B*: sulforaphane, the sulfur-containing moiety in broccoli. *C*: H_2S-releasing compound morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate (GYY4137). *D*: Lawesson's reagent. *E*: *S*-propargyl cysteine, a cysteine analog. *F*: anethole trithione hydroxide (ADT-OH), the dilthiolethione sulfur donor, is frequently added to a variety of compounds such as diclofenac (*G*) or valproate (*H*). *I, J*: methanethiosulfonate derivatives of valproate. *K–M:* other H_2S donors that have been combined with L-DOPA. ATB, Antibe Threapeutics (Hamilton, Canada); ACS, CTG-Pharma, (Milan, Italy).

Naturally occurring H_2S-donating drugs. The best characterized, naturally occurring H_2S-donating compound from garlic (*Allium sativum*) is allicin (diallyl thiosulfinate), which decomposes in water to a number of compounds. Two of these, diallyl disulfide and diallyl trisulfide (or DATS; Fig, 4*A*) are the most efficacious H_2S donors and readily vasodilate rat aortas (16). It should be noted garlic-mediated H_2S production by red blood cells and in buffer was determined under anoxic conditions, and H_2S production by rat aorta was measured at $P_{O_2} < 36$ mmHg. How these reactions proceed under more physiological conditions (e.g., P_{O_2}) remains to be determined.

Sulforaphane (Fig. 4*B*), 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). Sulphoraphane 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 h (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 whether either sulforaphane or erucin are metabolized to H_2S, or if erucin has any beneficial effects on the cardiovascular system.

Synthetic H_2S-donating drugs. A number of H_2S compounds have been synthesized with the intent of slowing the rate of H_2S release and thereby avoiding the transient H_2S surge characteristic of the sulfide salts. GYY4137 [morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate (Cayman Chemical, Ann Arbor, MI); Fig. 4*C*] (92) is a water-soluble molecule that is reported to slowly (over 90 min) release H_2S in acidic phosphate buffer. When GYY4137 was injected intrapertioneally or intravenously into rats, plasma H_2S increased from control 33 μmol/l to ~80 μmol/l in 30 min and was still elevated (50 μmol/l) 3 h later. By comparison, NaHS rapidly (within seconds) generated H_2S in buffer and did not affect plasma H_2S concentration when injected intravenously. GYY4137 produced a K_{ATP} channel-mediated relaxation of rat aortas and dilated the perfused kidney. In vivo, GYY4137 exhibits antihypertensive activity. Curiously, H_2S 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 H_2S was measured with an indirect and questionable methylene blue method (see *Separating Hype from Hope*).

Lawesson's reagent (Fig. 4*D*), another H_2S donor has been used with some success as an anti-inflammatory drug in the stomach (176).

Stimulation of H_2S production and the augmentation of H_2S-like effects by exogenous cysteine are well known. Several cysteine analogs 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. 4*E*. In a patent application (http://www.faqs.org/patents/app/20090036534) *S*-propargyl cysteine and *S*-allyl cysteine (50 $mg \cdot kg^{-1} \cdot day^{-1}$ ip for 7 days) was reported to increase plasma H_2S in rats with myocardial injury from 34.7 to 91.6 and 61.1 μM, respectively.

Additional benefits have been realized by complexing H_2S donors with other clinically efficacious drugs. The bulk of this work has successfully focused on blending the protective effects of H_2S with NSAIDs that of themselves often have adverse side effects in the gastrointestinal tract (174). Anethole trithione (ADT-OH; Fig. 4*F*), a dithiolethione, is one of the most commonly used H_2S donors. This molecule has been combined with numerous NSAIDs, including aspirin (ACS14; CTG-Pharma, Groton, CT), diclofenac (ATB-337; Antibe Threapeutics, Calgary, AL, Canada; ACS 15, S-diclofenac; Fig. 4*G*), indomethacin (ATB-343), mesalamine (ATB-429) and sulindac (S-sulindac). Dithioline derivatives of sildenafil (ACS6), valproate (ACS 2, S-valproate; Fig. 4*H*) and the anti-glaucoma drug latanoprost (ACS-67) have been developed, as have other methanethiosulfonate derivatives of valproate (ACS 33, Fig. 4*I* and ACS 43, Fig. 4*J*) (20, 126). A number of H_2S-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 AT_1 receptor inhibitor losartan (H_2S-EXP 3714) has been reported (104), but efficacy studies have not been published.

The effects of H_2S-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 μmol/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_2S. S-diclofenac also reduces plasma IL-1β/TNF-α, elevates plasma IL-10, and increases plasma H_2S concentration from 25 to 37 μmol/l at 45 min and to 33 μmol/l 6 h post injection (91). S-diclofenac perfusion (10 and 30 μM) protects against ischemia-reperfusion injury in the isolated rat heart, which is mediated partly by opening K_{ATP} channels, and possibly by increasing cysteine uptake and GSH synthesis. In this model, S-doclofenac also reduces creatine kinase and lactate dehydrogenase release and decreases the inhibitory effect of diclofenac on protective PGI_2 production (132). Intravenous injection of H_2S-releasing aspirin (ACS14; 0.1 mmol/kg) produces a slight transient rise in plasma H_2S [0.55 to 1.4 μmol/l that remains slightly elevated (0.62 μmol/l) at 150 min (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 and heart rate, but increases plasma GSH and 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). Intraperitoneal injection of ACS14 or ADT-OH (both 0.1 mmol/kg) increases plasma H_2S from 0.4 to ~0.65 μM in 15 min with a 2nd peak (0.5 μM) at 12–24 h, which is attributed to elevated plasma cysteine and subsequent metabolism to H_2S. 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_2S-donating sildenafil (ACS6) combines the inhibitory action of sildenafil on PKG with an H_2S inhibition of PKA in porcine pulmonary arterial endothelial cells. Collectively, these inhibit superoxide formation and $gp91^{phox}$ expression, suggesting ACS6 may be effective in treating adult respiratory distress syndrome. H_2S release from 10 μM NaHS peaks in 30 min, whereas H_2S release from 10 μM ACS6 peaks in 120 min. Furthermore, more H_2S is released from ACS6 than NaHS and only 25% mol/mol H_2S is released from either NaHS or ACS6. ACS6 release of H_2S 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_2S effect) and PKG (sildenafil effect). There is no specific vasodilatory benefit from H_2S released by ACS6 (142), which seems to be more or less typical for all synthetic H_2S-donating drugs.

Sodium sulfide (NaHS) and sodium sulfide (Na_2S) have long been used to generate H_2S. While these are frequently called "H_2S donors", and have even been reported to slowly release H_2S (111), they are sulfide salts, and when placed in water, their dissociation and subsequent H_2S formation are nearly instantaneous (84). Caution should be exercised with Na_2S, as this is a strong alkali. Na_2S 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. For recent reviews, see Refs. 9, 20, 104, 128, 153, 154, 173, and 205.

Separating Hype from Hope

There is little doubt that exogenous H_2S affects a myriad of physiological systems, and many studies have been corroborated to some extent by compounds that can theoretically alter endogenous H_2S production. Moreover, the beneficial results obtained with the H_2S-"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_2S chemistry—what can H_2S do? In their review, Sen et al. (137) state that "H_2S is a strong oxidant," and although H_2S is more commonly thought of as a strong reducing agent, Kabil and Banerjee (66) show it is a relatively weak reducing agent, especially compared with other intracellular thiols, such as glutathione. H_2S 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_2S inhibits oxidative phosphory-

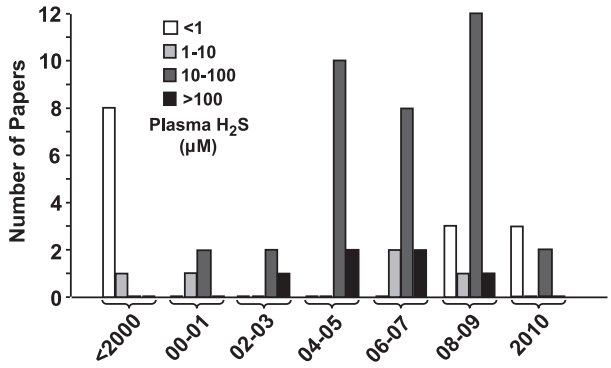

Fig. 5. Frequency distribution of papers reporting plasma H_2S at various concentrations. Prior to 2000, nearly all studies reported H_2S as undetectable (<1). Subsequently, the number of studies reporting H_2S between 10 and 100 μM has progressively increased (most between 20 and 40 μM), with a few studies reporting values in excess of 100 μM (most between 200 and 300 μM). [Modified from Olson (116).]

lation at ~20–40 μM (6, 24) and these concentrations were greatly exceeded in experimental conditions involving either exogenous H_2S or H_2S donors, it can be difficult to separate specific H_2S effects from general metabolic depression or even more general reducing and/or antioxidant actions.

Blood and tissue H_2S concentrations—what is real and what is an artifact? The overwhelming majority of studies and reviews on the biology of H_2S refer to "physiological" concentrations of 20–40 μM H_2S in blood (with some reports of plasma H_2S approaching 300 μM). In turn, 20–300 μM H_2S has been used to validate many "physiological" experiments. However, reports of plasma H_2S >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 H_2S levels >10 μM are unrealistic has been argued on the basis of both practical and methodological considerations (116). In addition, the use of newer methods, especially a polarographic (amperometric) sensor that directly measures H_2S gas, fails to find plasma H_2S approaching even 1 μM (186). There are also a number of other practical considerations, namely *1*) there is no odor of H_2S in plasma, which there should be even at 1 μM, *2*) H_2S 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 H_2S production at this level (41), *5*) H_2S is rapidly consumed by tissues in the presence of oxygen (Fig. 3) (82, 118, 138), and *6*) H_2S inhibits mitochondrial respiration at 20–40 μM. Furthermore, because H_2S is metabolized by the mitochondria as fast as it is generated under normoxic conditions, H_2S will increase only when tissue P_{O_2} falls.

In a recent study, Wintner et al. (188) examined kinetics of plasma H_2S 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 H_2S 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_2S 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 1 h of infusion of 20 $mg \cdot kg^{-1} \cdot h^{-1}$, 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_2S is measured with polarographic sensor, and a strong reducing agent, DTT, known to liberate sulfane sulfur, is added to blood before and after exogenous H_2S. As shown in Fig. 6, raising the concentration of DTT in buffer to 10 mM increased current flow equivalent to approximately one-fifth of the response produced by increasing H_2S concentration to 50 μM (Fig. 6*A*). A similar 10-mM increase in DTT

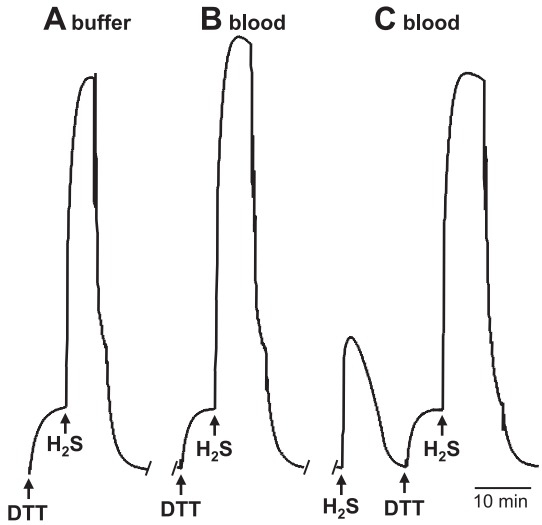

Fig. 6. Lack of evidence of sulfane sulfur in trout blood. *A*: DTT added to Cortland buffer produces a characteristic current in the polarographic H_2S sensor. The addition of H_2S increases current flow five-fold. *B*: similar additions of DTT and H_2S to heparinized trout blood produce identical responses. *C*: H_2S added to blood without prior DTT treatment rapidly disappears. The addition of DTT to H_2S-treated blood produces the same response as DTT addition to Cortland (*A*) or untreated blood (*B*) and subsequent addition of H_2S in the presence of DTT also produces the same response as H_2S (*A* and *B*). Each addition of DTT will produce a final DTT concentration of 10 mM, and each addition of H_2S will increase the final concentration by 50 μM (N. L. Whitfield, E. L. Kreimier, and K. R. Olson, unpublished data).

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_2S (Fig. 6*B*). When H_2S was added to untreated blood (Fig. 6*C*), the H_2S was rapidly consumed. The addition of DTT to the H_2S-spiked blood produced the same response as DTT addition to buffer or untreated blood, i.e., there was no evidence of liberation of H_2S from sulfane sulfur over the 10-min treatment period. A second addition of H_2S to the H_2S-DTT-treated blood produced a response similar to that produced by the addition of H_2S to the buffer or untreated blood. The consumption of H_2S by untreated blood and the inability of DTT-treated blood to consume H_2S suggest that H_2S 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_2S concentration from 41 to 18 μmol/l in CSE gene-deleted mice is really key evidence for H_2S as a "physiological" gasotransmitter of comparable importance to nitric oxide and carbon monoxide (42). Clearly, one must also question the numerous reports showing elevated (>10 μM) plasma H_2S concentrations achieved with the H_2S-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_2S concentrations and tissue production. Recent studies have shown that H_2S is consumed by tissues in the presence of oxygen, and H_2S production is only observed under hypoxic or anoxic conditions (41, 118). In fact, even exogenous H_2S is quickly and efficiently consumed by tissues at oxygen partial pressure (Po_2) greater than 10 mmHg (118). By comparison, water or tissue samples in equilibrium with room air typically have a Po_2 greater than 140 mmHg. In addition, unphysiologically high cysteine (usually 10 mM, compared with normal <1 mM) is used in tissue production studies, and serine and homocysteine are typically absent. This can artificially increase the rate of H_2S 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 H_2S production in cells in real time and under physiological conditions, other than showing an inverse relationship between H_2S production and Po_2. For recent reviews, see Refs. 24, 96, 116, and 163.

Does H_2S 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 H_2S can be accepted as a biologically relevant signaling molecule. Foremost among these is the discrepancy between tissue and plasma concentrations and the dose of exogenous H_2S needed to produce physiological responses. In addition, there is relatively little evidence regarding how H_2S 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 H_2S only transiently present in blood and tissue, and does it serve to initiate downstream effects that can be sustained for lengthy periods? Is H_2S merely a by-product 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_2S biosynthesis that have led us astray? If H_2S is the principal signaling moiety, can H_2S-donating drugs be targeted for tissue-specific H_2S release? It would seem that these are pressing questions that, if answered, may save considerable time and effort as we attempt to target H_2S pathways to treat the variety of diseases that have been implicated in H_2S pathophysiology. For a recent review, see Ref. 96.

Perspectives and Significance

H_2S 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_2S 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_2S 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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