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FORUM REVIEW ARTICLE

A Practical Look at the Chemistry and Biology of Hydrogen Sulfide

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Abstract

Significance: Hydrogen sulfide (H_2S) is garnering increasing interest as a biologically relevant signaling molecule. The effects of H_2S have now been observed in virtually every organ system and numerous physiological processes. ***Recent Advances:*** These studies have not only opened a new field of "gasotransmitter" biology, they have also led to the development of synthetic H_2S "donating" compounds with the potential to be parlayed into a variety of therapeutic applications. ***Critical Issues:*** Often lost in the exuberance of this new field is a critical examination or understanding of practical aspects of H_2S chemistry and biology. This is especially notable in the areas of handling and measuring H_2S, evaluating biosynthetic and metabolic pathways, and separating physiological from pharmacological responses. ***Future Directions:*** This brief review describes some of the pitfalls in H_2S chemistry and biology that can lead or have already led to misleading or erroneous conclusions. The intent is to allow individuals entering or already in this burgeoning field to critically analyze the literature and to assist them in the design of future experiments. *Antioxid. Redox Signal.* 17, 32–44.

Introduction

Fifteen years ago the pioneering studies of Kimura's group suggested that hydrogen sulfide (H_2S) was a biologically relevant signaling molecule (1). Since then, the field of H_2S biology has exploded, seemingly without much of the initial skepticism or reservation that accompanied its gaseous predecessor, nitric oxide (NO). Excitement over this molecule, from basic research to translational medicine, has spawned nearly as many reviews as primary research articles (*cf.* 17, 24, 32, 38, 44, 49, 55, 56, 68, 79). In many instances H_2S seems to fit the criteria for a "gasotransmitter" as adapted from the well-known criteria for neurotransmitters by Wang (74); *i.e.*, gasotransmitters must 1) be a small molecule of gas, 2) be freely membrane permeable, 3) be endogenously and enzymatically generated in a regulated manner, 4) have well-defined specific functions at physiologically relevant concentrations, and 5) act on specific cellular targets. The plethora of observations that exogenous H_2S affects virtually every organ system and tissue appears to bear this out. Not surprisingly, a variety of synthetic H_2S "donating" compounds have been synthesized and are currently being used in a number of clinical trials (55).

Surprisingly few reviews, however, have looked beyond the exuberance and critically examined the field "warts and all." In one seminal review, appropriately titled "Endogenous production of H_2S in the gastrointestinal tract: still in search of a physiologic function," Linden *et al.* (42) reviewed and modified Wang's five criteria for a gaseous signaling molecule; *i.e.*, it must 1) be a gas, 2) be endogenously and enzymatically generated in a regulated manner, 3) with exogenous application, cause a well-defined physiologic effect at physiologically relevant concentrations that mimics the effect of the endogenously produced H_2S on tissue activity, 4) act at specific cellular targets, as demonstrated by competitive antagonism, 5) employ a specific mechanism of inactivation. The authors then went on to show that (with emphasis on the gastrointestinal tract) H_2S fulfills the first criterion, partially fulfills the second criterion (a mechanism for regulating H_2S production has yet to be identified), partially fills the third criterion (the definition of "physiologically relevant concentrations" remains to be determined), partially fills the fourth criterion (competitive antagonism and receptor binding kinetics remain to be determined), and fulfills the fifth criterion

Innovation

H_2S biology is an exciting field that is attracting an ever-increasing number of investigators with wide and varied interests. This review emphasizes a number of areas that have either become erroneously entrenched in the literature or are especially problematic in working with this gas.

"Man is so intelligent that he feels impelled to invent theories to account for what happens in the world. Unfortunately, he is not quite intelligent enough, in most cases, to find correct explanations."—Aldous Huxley

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since tissue oxidation of H_2S is well known. The authors conclude that while H_2S is a promising candidate as a gasotransmitter, a number of questions remain to be answered.

The present review takes another critical look at H_2S chemistry and biology. The intent is to examine areas that have either been overlooked, generated confusion, produced conflicting opinions, or raised interesting and pressing questions. Clearly, not all areas in this expanding field can be covered in detail in this brief review, and I apologize for topics or significant articles that are not covered.

H_2S Chemistry

Hughes *et al.* (25) reviewed the practical aspects of preparing and working with H_2S under a variety of conditions. The following paragraphs emphasize related aspects of H_2S measurement and chemistry in physiological solutions and buffers (see also Table 1).

Measuring H_2S

A number of recent reviews have summarized the advantages and disadvantages of extant methods for measuring H_2S in buffers, biological fluids, and tissue samples (25, 53, 72), and the reader is referred to these for further details. This section focuses on the half dozen methods most frequently used for H_2S measurements in biological samples. Emphasis is placed on their limitations and potential for error.

The most commonly used method is the colorimetric generation of methylene blue by the reaction of H_2S with *N*,*N*-dimethyl-*p*-phenylenediamine sulfate. This method was developed for measuring H_2S in aqueous solutions and has more recently been adapted (generally inappropriately) to measuring H_2S in plasma and biological fluids in which it produces spuriously high values (53). In addition, the methylene blue method lacks sensitivity at low H_2S concentrations, and Hughes *et al.* (25) have recently shown that due to the formation of dimers and trimers of methylene blue, the absorption spectra of aqueous solutions of methylene blue do not obey Beer's law. In fact Beer's law only seems applicable at sulfide concentrations below 1 μM. These values are well below the 20–300 μM commonly reported in biological samples using this method. Because the methylene blue reaction proceeds under acidic conditions, it is not possible to separate free H_2S from acid-labile H_2S derived from iron–sulfur groups in cytochromes and other iron centers; the latter may be several thousandfold in excess of free H_2S (*cf.* 37). This method requires that the sample is mixed with analyte 20 to 30 minutes before the color is fully developed and the color intensity changes with time. The methylene blue method may be suitable in experiments in which there is no free protein, such as buffer-perfused organs. However, it cannot be used for continuous measurement of H_2S under physiological conditions, in real-time, or for simultaneous measurement of O_2.

Sulfide-specific ion-selective electrodes (ISEs) that measure S^{2-} have also been used on biological samples, but they too are not without error. Formation of S^{2-} requires a strong (pH > 11) alkaline solution, generally referred to as the "antioxidant buffer," to drive the equilibrium between H_2S, HS^-, and S^{2-} to favor S^{2-}. These alkaline conditions appear to promote hydroxyl replacement of cysteine sulfur thereby producing erroneously high sulfide concentrations that continue to increase with time (76). Like the methylene blue method, the ISE cannot provide information in real-time on unadulterated samples nor can it be used for simultaneous measurement of H_2S and O_2. Furthermore, the sensitivity appears to be insufficient for most biological samples.

Ubuka *et al.* (73) developed a method for measuring H_2S and acid-labile sulfide by initially trapping the evolved gas in an alkaline solution followed by gas chromatography with flame photometric detector and ion chromatography. They were able to measure acid-labile sulfide in tissues as low as 100 nmol/g tissue but did not detect free H_2S. The method is more sensitive than either methylene blue or ISE but cannot provide real-time measurements of H_2S in unadulterated samples.

The well-known method of measuring thiols by derivatization with excess monobromobimane (MBB) and subsequent measurement of the stable sulfide-diamine product with reverse phase high pressure liquid chromatography

Table 1. Common Methods for Measuring H_2S in Blood and Tissues

Method	*Sensitivity*	*Blood (μM)*	*Tissue (μM)*	*Interferences*	*Anoxia*	*Real-time*	*Subcellular*
Methylene blue	>1 μM	20–300	40–200	Protein, acid-labile sulfides, absorption spectra non-linear, time sensitive	yes	no	no
Sulfide ISE	>1 μM	20–300	20–300	Strong alkalinity required, time-sensitive liberation of sulfide from protein	no	no	no
GC-FPD, IC	low nM	nd	nd	High pH required, IC requires oxidation to sulfate	yes	no	no
MBB	low nM	0.1–1		Unknown sulfide "sink" in blood, tissue extraction suggested, reaction products stable	yes	no	no
Headspace, CG	low nM	0.01	low nM		yes	no	no
Polarographic (amperometric)	0.2 μM	nd–<1	nd–<1	Electrode sensitive to temperature, pressure, requires frequent calibration	no	yes	no
SSFD	>1 μM			Time delay, sensitivity	no	no	yes

GC-FPD, gas chromatography with flame photometric detector; IC, ion chromatography; ISE, ion-selective electrode; MBB, monobrombimane; SSFD, sulfide-sensitive fluorescent dye; nd, not detectable.

(HPLC) coupled with fluorescence detection has recently been used to measure plasma H_2S (63, 77). This method is described as being "suitable for sensitive quantitative measurement of free hydrogen sulfide in multiple biological samples such as plasma, tissue and cell culture lysates, or media" (63). Reagents must be made up in deoxygenated solutions and MBB must be protected from light. Maximum yield is obtained at a pH of 9.5 and at 1% oxygen or lower. The limit of detection is 5 nM sulfide, and the reaction product is reported to be stable. Real-time measurements of H_2S under normoxic, physiological conditions are not possible.

Analysis of H_2S evolution into headspace gas and subsequent gas chromatography has been used to measure both tissue and plasma H_2S in the nanomolar range (18, 37). By carefully controlling pH, Levitt *et al.* (37) were able to separate free sulfide (H_2S gas and HS^-) from acid-labile sulfide. Although this method can be used to measure H_2S in unadulterated biological samples, it is not capable of measuring H_2S in real-time. In addition, these determinations are usually performed under anoxic or severely hypoxic conditions with an extended period of time required for evolution of gas into the headspace.

The polarographic (amperometric) electrode, originally developed by Jeroschewski and Steuckart (29) and modified by Kraus and Doeller (34), operates similar to the standard Clark O_2 electrode and uses a relatively H_2S-gas–specific membrane and polarizing voltage. This method can be used with otherwise unadulterated samples of tissue homogenates or pieces of tissue, with cultured cells, and even in an extracorporeal loop for continuously monitoring plasma H_2S in unanesthetized animals (76). The polarographic electrode directly measures the concentration of dissolved H_2S gas in real-time and the electrode has a detection limit of 10 to 20 nM H_2S gas or ~100–200 nM total sulfide (76). Because this method only measures H_2S gas, total sulfide must be calculated from concomitant pH measurements. Polarographic electrodes also consume sulfide, albeit slowly. However, this can become problematic when measurements are made in a small volume in which there is an increased probability of lowering the total sulfide concentration, or in unstirred conditions in which electrode consumption can reduce sulfide concentration in the immediate area surrounding the tip of the electrode. Amperometric electrodes are generally pressure and temperature sensitive and prone to drift, necessitating frequent calibration. They can be used in the presence of oxygen and offer the particular advantage of being able to simultaneously compare oxygen and H_2S concentrations (56).

Wintner *et al.* (77) compared the amperometric response of the sensor to the MBB method. They observed that while both methods produced essentially identical responses when Na_2S (10 μM final concentration) was added to either HEPES buffer or human plasma, the amperometric sensor only detected a brief, transient increase in total sulfide when Na_2S was added to human whole blood, whereas the sulfide measured with the MBB method was more than three times that of the sensor and only slowly decreased over the following 30 minutes (Fig. 1). They attributed the differences between the two methods to the ability of the MBB method to detect a "reversible sulfide sink" in addition to free H_2S. This sink was not due to H_2S gas nor the common oxidation products of sulfide (sulfite, sulfate, or thiosulfate), and the authors proposed that it is a loosely bound moiety, still in the −2 oxidation state. They also suggested it was a form of a persulfide (RS_nH) on the cysteine residues of one or more proteins. It is unlikely that this sulfide sink is either acid-labile sulfide or sulfane sulfur because neither of these appears to be released by H_2S-doped whole blood (55). It remains to be determined whether or not this unknown sulfide can be released under physiological conditions and, if so, what mechanism is involved. If this is indeed a heretofore unidentified sulfide moiety it may help explain how the effects of exogenously administered H_2S persist long after the gas has disappeared.

Sulfide-sensitive fluorescent dyes, similar to the well-known ion-sensitive dyes such as the calcium reporter Fura-2 are currently being developed (43, 60). These have the potential to resolve many of the key questions regarding H_2S signaling mechanisms and effector pathways. To date, however, they appear to lack the sensitivity to measure endogenous H_2S at submicromolar levels and in real-time.

Sources of H_2S

The two most common sources of H_2S are the sulfide salts, sodium hydrogen sulfide (NaHS), and sodium sulfide (Na_2S).

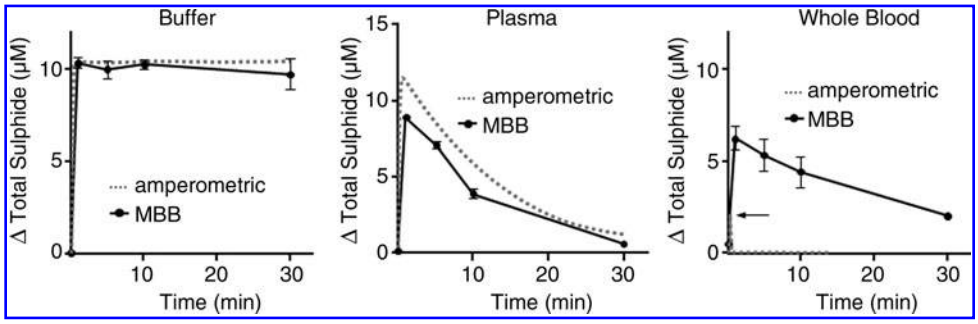

FIG. 1. Comparison of H_2S measured in buffer, human plasma, and human whole blood with the amperometric H_2S sensor and the monobrombimane (MBB) method. Na_2S (final concentration 10 μM) was added to HEPES buffer (50 mM HEPES, 155 mM NaCl, 3 mM glutathione, 1 nM diethylenetriaminepentaacetic acid, pH 8.0), human plasma, or human whole blood. Both methods produce similar results in HEPES buffer and plasma. However, in whole blood there was only a transient spike in sulfide when measured with the amperometric sensor, whereas more than three times as much sulfide was measured with the monobromobimane (MBB) method and sulfide concentration remained elevated for at least 30 minutes. Redrawn from Wintner *et al.* (77), with permission.

These compounds are frequently and erroneously called H_2S "donating compounds" and it has been reported that they slowly release H_2S when dissolved (47). However, other studies (13, 39) have shown that when either Na_2S or NaHS is dissolved, H_2S is formed essentially as fast as crystal solvation. The major difference between the study of Muzzaffar *et al.* (47) and those of DeLeon *et al.* (13) and Li *et al.* (39) was that the former measured sulfide with the methylene blue technique, whereas the latter two studies measured H_2S gas in real-time with polarographic electrodes. Calcium sulfide (CaS) has also been used as a H_2S "donor" (41), but this does not appear to convey any advantage over the other sulfide salts and, although not measured, CaS presumably forms H_2S upon solvation at the same rate as NaHS and Na_2S. H_2S gas can also be passed through distilled water or buffers but this is far less convenient and often considerably more hazardous (25).

A number of true H_2S "donating" drugs are also available (5, 45, 55, 59). These reportedly have the advantage of a slower and more sustained release of H_2S than the sulfide salts, and they can be combined with other drugs to achieve multiple therapeutic effects. Some of these drugs also require an enzymatic process to liberate H_2S, which should enhance H_2S delivery to the target cells; *i.e.*, ACS6 release of H_2S is approximately four times greater when incubated with endothelial cells than in buffer (47). Rarely has the release of H_2S from these compounds been measured in real-time. Li *et al.* (39) reported a slow release of H_2S from GYY4137 [morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate] in buffer when measured with the polarographic electrode; however, the release of H_2S by GYY4137 in blood was inexplicably measured with the methylene blue method and not with the polarographic electrode. This makes any direct comparison difficult.

pH

Dissolved H_2S is a weak acid in equilibrium: $H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + H^+$. Only the first reaction is relevant for biological samples because the pK_{a1} is in the physiological range. The pK_{a2} for the second reaction is over 12, with some reports suggesting it is as high as 19 (25). For all practical purposes, S^{2-} is insignificant. Both pKs vary with temperature and salinity; the effect of temperature on pK_{a1} can be estimated from the equation: $pK_{a1} = 3.122 + 1132/T$, where T is degrees Kelvin (52). This is not insignificant because the pK_{a1} changes from 6.98 at 20°C to 6.77 at 37°C. A 1 mM solution of NaHS or Na_2S at pH 7.0 will have 490 μM dissolved H_2S at 20°C and at 37°C the dissolved H_2S will be 372 μM, 25% less. A 1 mM NaHS or Na_2S solution at pH 7.4 will have 277 μM dissolved H_2S at 20°C and at 37°C the dissolved H_2S will be reduced by 30% to 191 μM. Thus, failure to correct for temperature can account for as much as a 30% error in estimating the amount of dissolved H_2S under physiological conditions.

H_2S-forming salts require protons from the solvent, and their solvation can have a substantial effect on the pH as it alkalinizes the solution. This can become especially problematic for solutions of Na_2S in which solvation requires two protons to produce H_2S; NaHS only requires one proton. Dombkowski *et al.* (15) and DeLeon *et al.* (13) have shown that the buffering capacity of many physiological buffers begins to fail around 1 mM sulfide. In Krebs–Henseleit buffer, commonly used in mammalian preparations, 1 mM Na_2S increases the pH by nearly one-half unit and 10 mM Na_2S increases it by over 2 pH units. As expected, this is considerably greater than that produced by NaHS for which a 10 mM solution increases the pH by a little over 0.5 pH units. It is not always clear whether studies that employ millimolar concentrations of sulfide salts titrate their solutions prior to use.

Oxygen

It is well known that H_2S is oxidized in solution especially in the presence of metal catalysts (9) and biological tissue (57) (discussed in a later section). Spontaneous H_2S oxidation is also pH dependent (8). As shown in Fig. 2, the rate of spontaneous H_2S oxidation increases nearly tenfold over the range of commonly encountered physiological pH (6.5–8.0). Since it appears that much of this spontaneous oxidation of H_2S is due to metal catalysts, especially ferric iron, that exist as impurities in the water, removing them with a chelator such as diethylenetriaminepentaacetic acid (DTPA) will greatly delay spontaneous oxidation. Hughes *et al.* (25) found that in the absence of DTPA, HS^- absorbance was halved in 3 hours, which argues against the rapid oxidation suggested by Wintner *et al.* (77); whereas, in the presence of 100 μM DTPA it did not change over this period. Of course ion chelation can also remove essential minerals when done in the presence of tissues. However, volatilization of H_2S, as described in the following section, appears to contribute far more to spontaneous H_2S loss than oxidation in a variety of biological experiments.

Volatilization

Because H_2S is a gas, the concentration of H_2S in solution can also be affected by volatilization, and the rate of H_2S evolving from solution is proportional to the surface area at the air–liquid interface. This is especially problematic in most, if not all physiological experiments because the tissues require continuous oxygenation and carbon dioxide must be eliminated. In tissue culture this is usually accomplished with a

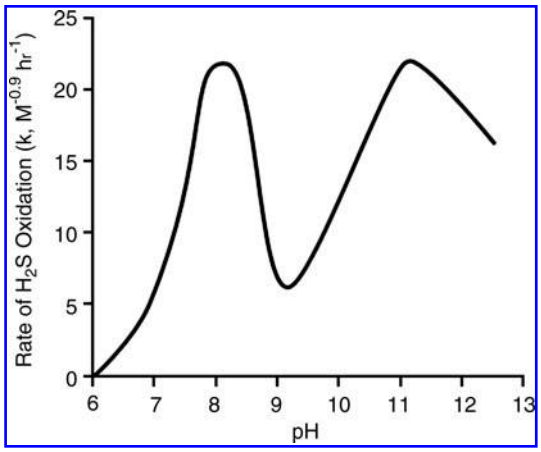

FIG. 2. The effect of pH on the rate of spontaneous H_2S oxidation. At pH < 6 H_2S oxidation is nil, it then reaches a maximum at pH 8 before falling to a nadir at pH 9. Over the range of physiological pH, ~6.5 to 8.0 the rate of H_2S oxidation increases approximately tenfold. Modified from Chen and Morris (8).

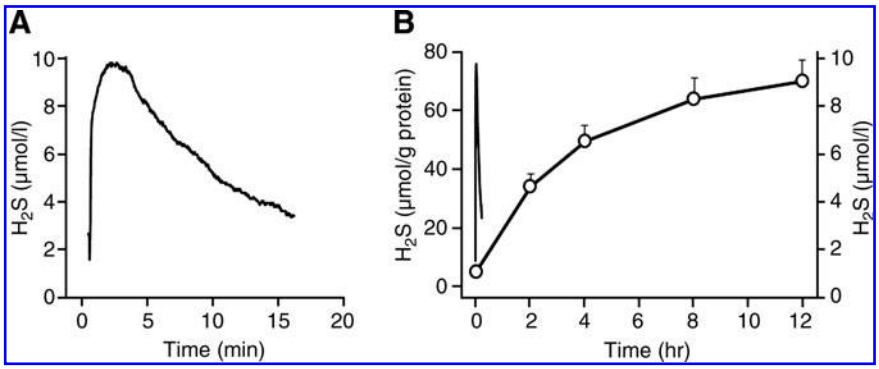

FIG. 3. Comparison of the rate of 10 μM H_2S lost from cell-free 24-well plates (μmol/L) to the rate of H_2S accumulation by confluent U118 cells in 24-well plates (μmol/g protein). (A) H_2S is lost exponentially with a halftime of $\sim$5 minutes from 24-well plates when measured continuously with the H_2S polarographic electrode. **(B)** U118 cells accumulated H_2S (open circles) measured by the direct methylene blue method; transient line without symbols re-drawn from panel A. A, B; cell-free well plates redrawn from DeLeon *et al.* (13). B; H_2S accumulation by U118 cells redrawn from Lee *et al.* (36).

large surface/volume ratio and a monolayer of cells. With isolated organs or tissues, additional gassing is required and this often entails bubbling the tissue with some mixture of air, oxygen, and carbon dioxide, or circulating the buffer through an oxygenator. Control studies on the rate of H_2S volatilization as a function of the oxygenation process should be used to correct for H_2S loss. While H_2S volatilization is obvious to anybody working in this field, the impact of volatilization on the concentration of H_2S in biological experiments seems to have been overlooked.

To examine the potential for H_2S volatilization under typical laboratory conditions, we (13) measured the concentration of H_2S gas in buffer in real-time with a polarographic electrode under three experimental conditions commonly used in cardiovascular studies, tissue culture well plates in which gases exchange passively across the well surface, muscle myograph baths that are bubbled via a glass frit, and the Langendorff perfused heart apparatus in which buffer drains down a long glass column that is gassed.

As shown in Fig. 3A, there is an exponential loss of H_2S from 24-well tissue culture plates containing a physiological buffer. The rate constant (k) for H_2S volatilization is 0.13 min^{-1} and the half time ($t_{½}$, the time for H_2S concentration to be halved) is 5 minutes ($t_{½} = 0.693/k$). Because this process is passive, the rate constant and half time are independent of the initial H_2S concentration. This means that 13% of the H_2S remaining in the well is lost every minute and after 30 minutes only 2% of the initial H_2S will be left in the well. Thus, volatilization becomes problematic when exposing cells to H_2S. Lee *et al.* (36) exposed THP-1 and U118 cells to 10 μM NaHS in 24-well plates in 1 mL of Dulbecco's modified Eagle medium nutrient mixture F12 containing 5% fetal bovine serum and hydroxylamine (to inhibit endogenous H_2S synthesis). They measured H_2S in the cells by the direct methylene blue method and reported continual H_2S accumulation for 12 hours (Fig. 3B). According to the rate constants for H_2S volatilization from our study, the predicted concentration of H_2S after 12 hours should be around 4×10^{-43} μM. Although the methylene blue method liberates acid-labile as well as free sulfide, the amount of acid-labile sulfide is not expected to accumulate during the duration of the experiment. This raises the question of what is being measured with the methylene blue method. Clearly, Lee *et al.* (36) show something is increasing, as have numerous other experiments (reviewed by Olson [55]). It is doubtful if this is either endogenous or exogenous H_2S. It remains to be determined if there is a different endogenous sulfide that is being measured with the methylene blue method, or if the molecule(s) being measured is some as yet unidentified storage form of H_2S. It is also possible that this is merely an artifact.

H_2S is lost from bubbled muscle myographs ($k < 0.2$ min^{-1} and $t_{½}$ < 4 min) three times faster than from unbubbled myographs and also faster than from the 24-well tissue culture plates (Fig. 4A) (13). Presumably this is due to the additional air/liquid surface area from the fine bubbles. The rate of H_2S lost from myographs bubbled with 100% pure oxygen was slightly but significantly slower than when bubbled with 100% nitrogen. This shows that H_2S oxidation is insignificant within the typical 30-minute time frame of many experiments. It also suggests that there may be some, as yet unidentified, chemical or physical interaction between H_2S and N_2 or at the air–bubble interface. Because the response time of isolated blood vessels is typically 1–5 minutes, and H_2S is added in 10- to 20-minute intervals in cumulative dose–response experiments, there is little chance that the vessels will ever experience the desired H_2S concentration. In fact, when H_2S is added to the myograph and monitored with the polarographic electrode, the peak H_2S concentration measured is typically < 90% of the predicted concentration. In a dose–response study on mouse aortas bubbled with 95% O_2/5% CO_2, Al-Magableh and Hart (2) only measured 91 μM H_2S with a polarographic H_2S sensor in myographs after cumulative additions of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M NaHS. They hypothesized that this discrepancy was due to differences between H_2S measured by the sensor and the actual dissolved NaHS. However, it is likely that volatilization was a major factor in their observations.

H_2S volatilization from the Langendorff perfused heart apparatus is the fastest (Fig. 4B). When injected into the pump effluent, H_2S concentration transiently increases. However, in the time for one complete circulation (1 minute in this preparation) H_2S concentration had fallen to < 20% of the desired concentration, at 2 minutes it was < 10%, and it continued to decline exponentially thereafter. Half-times for the transient spike were < 6 seconds and the remaining H_2S disappeared with half times of < 3 minutes.

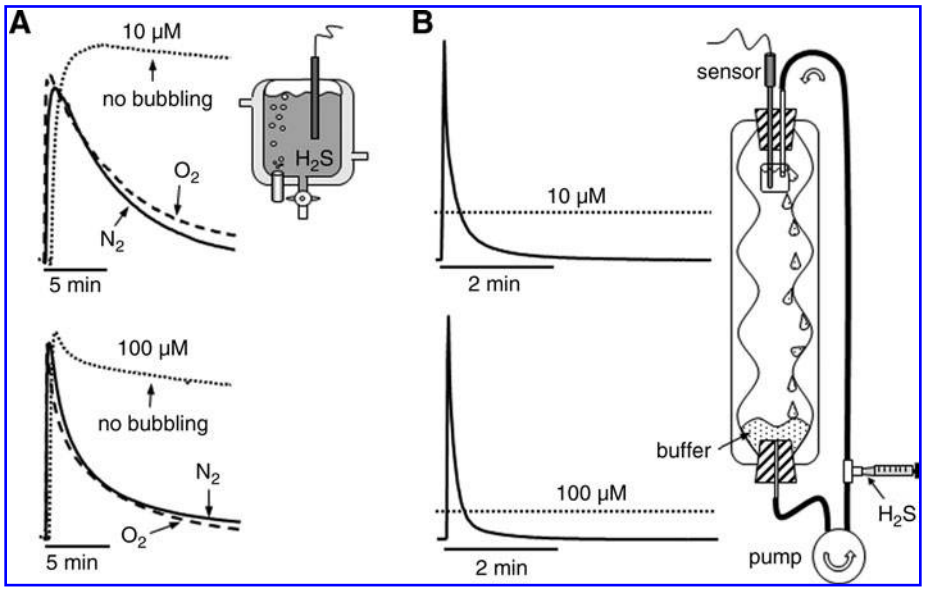

FIG. 4. Real-time measurement of 10 and 100 µM H_2S volatilization from a muscle myograph (A) and Langendorff perfused heart apparatus (B). (A) In myographs, volatilization is slowest from unbubbled myographs and fastest when bubbled with 100% nitrogen (N_2). **(B)** in the Langendorff apparatus H_2S is pumped into a 10-mL beaker (simulating the heart) containing the polarographic H_2S sensor, and it then flows down the side of the apparatus where gas is equilibrated. A bolus of H_2S is injected into outflow from the pump and produces a transient increase in H_2S. By the time the bolus makes the second pass across the sensor (1 minute) the measured H_2S concentration is well below predicted values (dotted lines). Modified from DeLeon *et al.* (13).

Rapid volatilization of H_2S creates several problems in designing and interpreting experiments. First, it is evident that the desired H_2S concentration is never attained because the outgassing process occurs immediately upon sample addition. Second, the rapid fall in H_2S concentrations that results from bubbling renders H_2S dose–response determinations rough estimates at best. Third, where the effects of H_2S are demonstrated for hours (or days) after a single treatment it is not clear if this is an actual response to H_2S. Furthermore, it is also unclear if a metabolite or decomposition product of H_2S produces the observed response or if H_2S initiates a long-lived signal cascade. Nevertheless, it seems likely that many of the dose- dependent effects of H_2S and dose–response curves that have been published in the literature need to be revisited as they most likely overestimate the actual tissue exposure. These studies also help explain, in part, why the concentrations of exogenous H_2S needed to produce biological responses are considerably greater than recent estimates of endogenous H_2S in blood or tissue.

H_2S as an oxidant/reductant

H_2S is often regarded as a strong antioxidant (38, 62). Exogenous H_2S has been shown to reduce hypochlorous acid (48) and is likely to reduce peroxynitrite (75), superoxide (7), and hydrogen peroxide (19), and H_2S has been proposed to effectively prevent oxidant injury in the brain (reviewed by Kimura [33]). While there is little doubt that H_2S is a reductant, how effective it is intracellularly remains to be determined. In order to demonstrate antioxidant activity in biological preparations the concentrations of H_2S typically are (or exceed) 100 µM *in vitro* (*cf.* 80). *In vivo*, an intraperitoneal injection of 5 mg/kg NaHS (20) and assuming distribution throughout the entire body water, will raise intracellular H_2S to ~140 µM. It remains to be determined if endogenous H_2S is sufficiently concentrated in cells to have a similar physiological effect. Furthermore, Kabil and Banerjee (30) point out that H_2S is a relatively weak reducing agent, especially when compared with other intracellular thiols such as glutathione, and Carballal *et al.* (6) suggest that the protective effects H_2S cannot be completely accounted for by direct reactions with oxidants.

H_2S has also been suggested to increase oxidative activity in cells by inhibiting oxidative phosphorylation and thus favoring superoxide formation. For instance, Eghbal *et al.* (16) showed that exogenous H_2S (200–400 µM) dose-dependently stimulated the formation of reactive oxygen species (ROS) in isolated hepatocytes. This concentration is considerably greater than that required to inhibit oxidative phosphorylation, which is usually reported to be ~20–40 µM (3, 11). It will be necessary to determine if these concentrations are approached under physiological conditions. It is also important to note that the H_2S concentrations used in most experiments are considerably greater than 40 µM and these may either provide excessive reductant or superoxide to the cellular milieu making it difficult to separate H_2S-specific effects from general metabolic depression or pharmacological reducing and/or antioxidant actions.

This also raises an interesting conundrum, if endogenous H_2S production increases to the point of inhibiting oxidative phosphorylation and stimulating ROS production, can these ROS then oxidize the H_2S? Could this be a cellular feedback mechanism for limiting H_2S concentration?

Alternative "forms" of H_2S

In addition to dissolved H_2S and HS^-, reduced sulfide can be associated with iron-sulfur clusters in an acid-labile

Table 2. Comparison of the Known Effects of H_2S Exposure to the Theoretical Equivalent Plasma Sulfide Concentration Assuming Equilibration Across Alveolar Membranes and No Blood or Tissue Metabolism

Ambient H_2S (ppm)	*Equivalent total plasma sulfide (μM)*[a]	*Effects*
0.01–0.3	0.004–0.1	Threshold for detection
1–3	0.4–1	Offensive odor, headaches
10	4.5	8-hour occupational exposure limit in Alberta, Canada
15	6.7	15 minute exposure limit in Alberta, Canada
20–50	9–22	Eye and lung irritation
100	45	Olfactory paralysis
250–500	112–225	Pulmonary edema
500	225	Sudden unconsciousness (“knockdown”), death within 4- to 8-hours
1000	450	Immediate collapse, breathing ceases within several breaths

This table shows that the majority of plasma and tissue H_2S concentrations reported in the literature (20–300 μM) would range from noxious to fatal and all would be malodorous.

[a]All except equivalent total plasma sulfide column modified from Guidotti (22). Equivalent plasma sulfide is the theoretical sulfide concentration in plasma calculated after Whitfield *et al.* (76) (supplemental information) with the following assumptions: 1) H_2S freely equilibrates across the alveolar membranes (26, 71), 2) Henry’s Law constant for H_2S at 37°C and 140 mM NaCl is 0.0649 $M \cdot atm^{-1}$ (12), 3) 20% of total sulfide exists as H_2S gas (52), and 4) there is no H_2S metabolism in blood or tissues. Table modified from Olson (55), with permission.

form and as persulfides, *i.e.*, sulfane sulfur (RS-S). The term acid-labile is derived from the observation that sulfide can be released from these clusters when pH falls below 5.4 (27). Sulfane sulfur appears to be the only product of the tandem enzyme pathway involving the enzymes cysteine aminotransferase and 3-mercaptopyruvate sulfur transferase (3-MST) (30) and has been proposed to be the major pathway for sulfane sulfur (and potentially H_2S production) by the brain (27, 64). H_2S can be liberated from 3-MST–sulfane sulfur by the ubiquitous reductant, thioredoxin, and by dihydrolipoic acid, both present in cells (46). Both acid-labile and sulfane sulfur have been suggested to serve as a relatively stable form of H_2S for intracellular storage and as a site from which H_2S can be readily mobilized. These possibilities are considered in the following sections.

H_2S biology

H_2S in blood

At present there is no consensus regarding the concentration of H_2S in either blood or tissues, and this must be resolved in order to differentiate between physiological and toxicological effects of exogenous H_2S. The overwhelming majority of H_2S concentrations reported in blood of vertebrates since 2000 are in the range of 20–40 μM, and there are at least a half dozen reports of plasma H_2S in excess of 100 μM. Arguments against plasma H_2S in the micromolar range have been summarized in several recent reviews (53, 55) and include the following. First, high blood H_2S concentrations are generally associated with methods that were not developed for use with biological tissue, most often methylene blue method and sulfide ISEs. A good example of this is the revision of plasma H_2S concentration in mice which the original study (78) reported to be 40 μM H_2S in plasma when measured with the methylene blue method. This was subsequently revised down to 1.6 μM by the same group using the MBB method (63). However, even micromolar concentrations are not typically observed when measuring H_2S with polarographic electrodes or with some of the more modern methods employing gas chromatography and HPLC. Whitfield *et al.* (76) observed barely to nondetectable levels of H_2S in blood plasma from a variety of animals using the polarographic electrode and Levitt *et al.* (37) reported 0.07 μM H_2S in mouse blood using HPLC analysis of headspace gas. Novel methods or refinements of existing ones will be needed to measure H_2S in blood or plasma, if it exists at all. Second, there is no odor of H_2S in plasma even though the human nose can smell 1 μM in buffer (Table 2). In fact, 45 μM H_2S in plasma would be equivalent to breathing 100 ppm H_2S (Table 2) and this should cause eye and lung irritation and even begin to paralyze the olfactory system. Pulmonary edema appears at 100 μM H_2S. Clearly, these concentrations are unphysiological. Third, H_2S readily equilibrates across respiratory surfaces and, if it existed in plasma, should be readily exhaled. Rapid appearance of H_2S during administration of Na_2S (IK-1001) has been observed in exhaled air from rats (26) and humans (71). As shown in Fig. 5, H_2S began to appear in exhaled air within 5 seconds after initiation of intravenous H_2S infusion in rats and H_2S concentration oscillated with each breath. When infusion was stopped H_2S concentration began to fall by the second breath and was halved within 3 seconds. Similar results were observed when a bolus of H_2S was injected in humans, although with a longer circulation time and longer onset and recovery periods. Toombs *et al.* (71) also observed that H_2S in exhaled air from humans prior to Na_2S injection was some 20 ppb greater than room air (6 ppb) indicative of continuous H_2S excretion. If this is the case, and with efficient transpulmonary H_2S exchange, this would imply that plasma H_2S is $\sim$6 nM, clearly well below the resolution of blood H_2S assays. Fourth, with the assumption that H_2S equilibrates across respiratory membranes, Furne *et al.* (18) calculated that there is not enough sulfur in the body to sustain H_2S production for even 24 hours. Fifth, H_2S is rapidly removed from the circulation by tissues. Norris *et al.* (51) have shown that the buffer-perfused rat liver removes nearly 97% H_2S at a perfusate concentration of 150 μM H_2S and 50% clearance at 300 μM H_2S. Sixth, H_2S has been shown to be rapidly consumed by tissues in the presence of oxygen (Fig. 6A) (35, 56, 57). Thus, it is unlikely that there is even enough H_2S escaping from normoxic tissue to contribute to plasma levels. If H_2S does exist in the plasma,

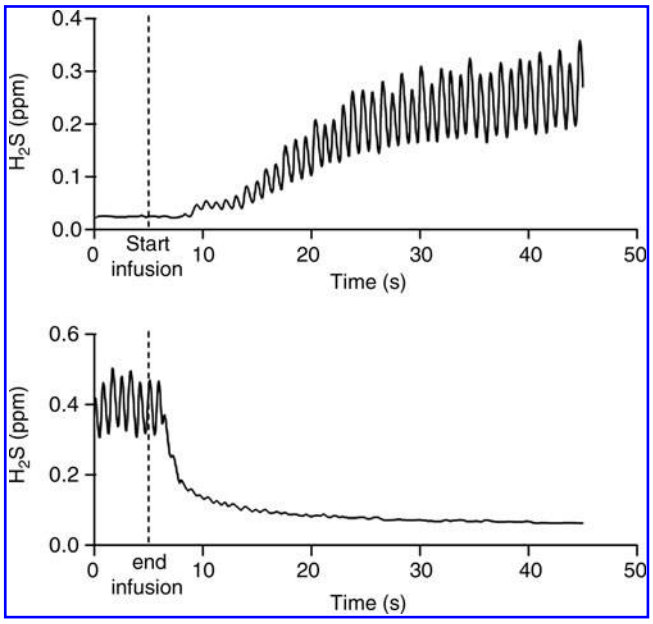

FIG. 5. Appearance of H_2S in exhaled air from rats infused intravenously with 0.6 mg/kg/min Na_2S. Upper trace shows that H_2S appears within 5 seconds after the onset of infusion and thereafter oscillates with each breath. At the end of infusion (lower trace) the concentration of H_2S falls within the first few breaths and is halved within 3 seconds. Redrawn after Insko *et al.* (26), with permission.

especially at levels sufficient to be considered physiologically relevant, which seems doubtful, there has yet to be a method that is sensitive enough to detect it.

It has been suggested that H_2S may be transported in the blood either as acid-labile sulfide, sulfane sulfur, or some other reversible "sink" (77), and Levitt *et al.* (37) reported ~1 μmol acid-labile sulfide in mouse blood. However, blood pH must fall below 5.4 to release H_2S from an acid-labile pool (27), which is highly unlikely. Furthermore, because blood is an oxidizing environment (50), it is difficult to imagine reducing conditions sufficient to release sulfide from a persulfide. Experiments designed to examine these possibilities have not supported either sulfur store. Acidifying mouse blood below 5.5 with trichloroacetic acid only produced 2.5 μM/kg whole blood measured with gas chromatography of headspace gas (37) and addition of 1 or 10 mM dithiothreitol to trout blood did not generate H_2S either in fresh blood or from blood primed with 10–100 μM H_2S (55). There are other possibilities as well; H_2S may be conveyed in the plasma in some other as yet unidentified form as proposed by Wintner *et al.* (77), although there is no evidence for this at present. It is also possible that H_2S is produced in blood. Searcy and Lee (61) demonstrated low levels of H_2S production by human red blood cells upon addition of elemental sulfur (S_8), but it is doubtful if this has physiological significance. Both cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) have recently been shown to be secreted by the liver and endothelial cells, circulate in an active form in human plasma, and are able to generate H_2S from cysteine or homocysteine plus cysteine (4). These authors (4) suggested that local H_2S production could protect the endothelium from elevated homocysteine. It remains to be determined if these plasma enzymes could also contribute to plasma H_2S.

H_2S in tissue

The vast majority of studies reporting the concentration of H_2S in tissues, or the rate of H_2S production by tissues, have also employed either the methylene blue method or ISEs and have been performed under relatively unphysiological conditions. Arguments can be made against these findings on both experimental and theoretical grounds (18, 37, 55).

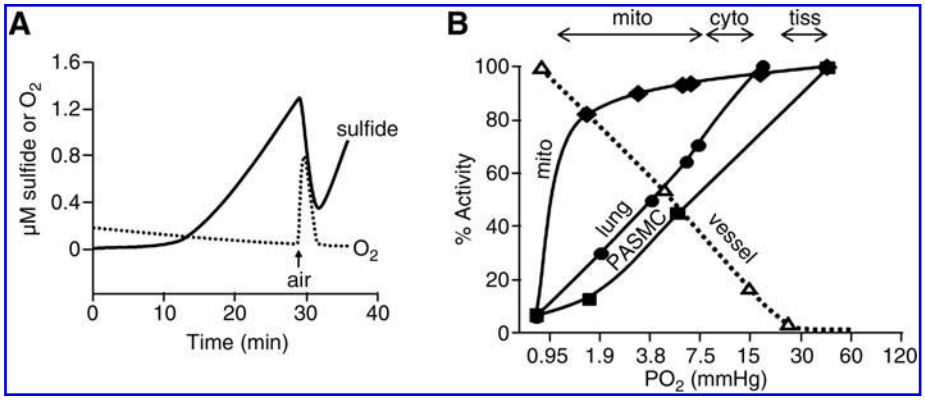

FIG. 6. Inverse correlation between H_2S and O_2 in biological tissues. (A) Simultaneous measurement of H_2S and O_2 concentrations in homogenized rat lung measured in real-time with H_2S and O_2 polarographic electrodes. H_2S is produced in the presence of precursors of H_2S biosynthesis, cysteine and α-ketoglutarate, and in the absence of O_2. Injection of a small air bubble (arrow) increases O_2 concentration and immediately decreases H_2S concentration. H_2S production resumes after the O_2 is consumed. Modified from Olson and Whitfield (56); **(B)** Solid lines show O_2-dependent 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; dashed line). Percent activity refers to the degree of H_2S consumption (100% = all H_2S consumed) or percentage of hypoxic contraction (100% = maximum vessel contraction). Modified from Olson *et al.* (57). Horizontal arrows above the figure refer to the range of Po_2 in mitochondria (mito), cytosol (cyto), and tissues (tiss) reported in the literature. Half maximal H_2S consumption and vessel contraction occurs at approximately the same Po_2 and these Po_2s are below those encountered in normoxia, *i.e.*, hypoxic conditions.

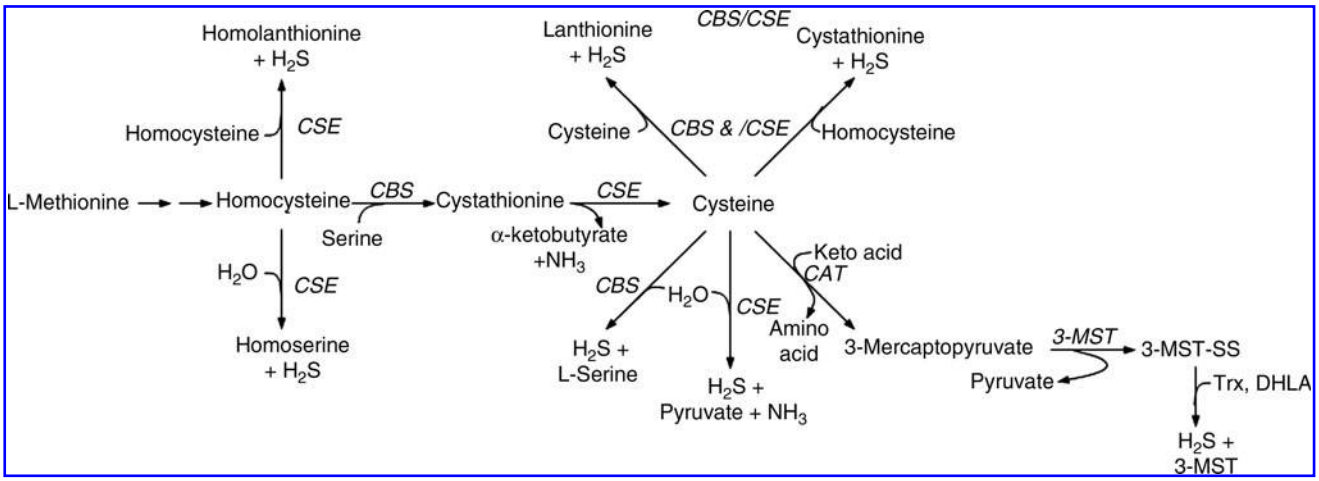

FIG. 7. Pathways of H_2S biosynthesis (see text for details). DHLA, dihydrolipoic acid; CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-ligase; ST, sulfur transferase; TR, thiosulfate reductase; Trx, thioredoxin; 3-MST, 3-mercaptopyruvate sulfur transferase.

First, there is the potential that the acidic conditions required for the methylene blue assay generate additional sulfide from acid-labile stores, as described for blood. In fact, one recent study suggested that acid-labile stores in a variety of mouse issues may be anywhere from 10,000 to 40,000 times the concentration of free H_2S (37). Second, these experiments are routinely conducted under anoxic or very hypoxic conditions. This not only prevents the rapid and effective oxidation that normally occurs under physiological conditions but also increases the reducing potential of the milieu and can potentially liberate sulfane sulfur. Third, these assays are generally conducted with unphysiologically high cysteine concentrations (typically 1 or 10 mM). This excess cysteine could artificially increase the rate of H_2S production by shuttling sulfur metabolism through normally minor metabolic pathways (66). Fourth, other important substrates for H_2S production, such as homocysteine, are not typically provided in the reaction mixture and, under physiological conditions, these may be more important than, or as necessary as, cysteine for H_2S production (Fig. 7). For example, the condensation of cysteine and homocysteine may account for 25%–70% of the H_2S generated under more physiological conditions (10, 30, 65). Failure to have sufficient homocysteine in the medium can also bias interpretation of the enzymes involved in H_2S biosynthesis. H_2S generation from cysteine is primarily catalyzed by CSE, whereas H_2S production from the condensation of cysteine and homocysteine is accomplished by CBS. In a recent study, Kabil *et al.* (31) further demonstrated the effect of substrate concentration on H_2S production by liver; at saturating cysteine and homocysteine concentrations (20 mM) H_2S production was similar for CBS and CSE, whereas at physiological levels of substrate and enzymes, CSE activity accounted for 97% of the H_2S produced. Furthermore, CBS activity can be regulated by a variety of factors that are not included in the medium such as *S*-adenosylmethionine, a well-known allosteric regulator of CBS. This can also bias both the rate and pathways of H_2S biosynthesis. It should also be noted that these enzymatic studies are generally performed under anoxic conditions (*cf.* 31). Thus, while providing valuable information on enzymatic activity and important substrates for H_2S production, they do not provide information on tissue H_2S production under truly physiological conditions. Clearly additional studies that replicate the cellular environment are necessary to both determine the rate of H_2S biosynthesis as well as the enzymes involved. Fifth, compounds frequently employed to inhibit either CSE (propargyl glycine and β-cyanoalanine), CBS (aminooxyacetate), or both (hydroxylamine) and thus used to distinguish the rate and/or pathway of H_2S production are not specific for these enzymes and they are often poorly absorbed by tissues (67). Sixth, although limited in number, a number of studies have suggested that free H_2S in tissues is under 50 nM (18, 37) or undetectable (73). Some of the potential sources for error in H_2S chemistry and blood/tissue measurements are summarized in Fig. 8.

Tissue H_2S metabolism

Tissue H_2S oxidation also argues against high blood and tissue H_2S concentrations. Essentially all measurements of H_2S production using the methylene blue, MBB, headspace gas methods, or S^{2-} ISEs have been performed in closed containers under anoxic or very hypoxic conditions. A number of studies have noted that H_2S production is increased or enhanced under hypoxic or anoxic conditions (14, 18) or that oxygen (O_2) consumption is increased by H_2S (21, 35). In fact, when measuring H_2S in headspace gas, Furne *et al.* (21) not only observed very low concentrations of H_2S in tissues when measured under hypoxic conditions, but they also observed that there was net consumption of H_2S by tissues in the presence of O_2. To my knowledge only one study has simultaneously measured H_2S and O_2 concentrations in tissues in real-time, and this study (57) demonstrated that H_2S is consumed by tissues in the presence of oxygen and H_2S production is only observed under anoxic or severely hypoxic conditions (Fig. 6A). In fact, even exogenous H_2S is quickly and efficiently consumed by tissues at oxygen partial pressures (Po_2) >10 mm Hg (Fig. 6B) (57). By comparison, water or tissue samples in equilibrium with room air typically have a Po_2 >140 mm Hg. Furthermore, tissue hypoxia is expected to increase the reducing conditions in the cell; this could liberate H_2S from persulfides (27), or the acidosis that routinely accompanies hypoxia could release acid-labile sulfur.

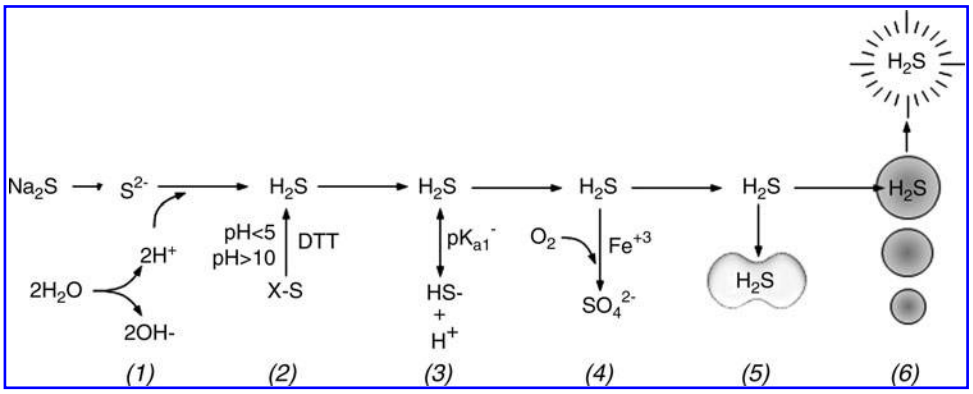

FIG. 8. Potential sources for error in H_2S chemistry and blood/tissue measurements. (1) Solvation of sulfide salts consumes one, or as shown here, two protons thereby alkalinizing the medium. (2) Analytical procedures that require strong acid (pH < 5), strong base (pH > 10) or reducing agents, such as dithiothreitol (DTT) can liberate H_2S from a variety of organic and inorganic sulfides (X-S). (3) Equilibrium between $H_2S \leftrightarrow HS^-$ is sensitive to pH and the pK_{a1} for this equilibrium is temperature sensitive. (4) H_2S is readily oxidized in the presence of metal catalysts such as ferric iron. (5) H_2S is rapidly removed from plasma by red blood cells (RBCs) or tissue. (6) H_2S is rapidly lost through volatilization when experiments are aerated or across the lungs during respiration.

H_2S in tissue and H_2S infusion, a quantitative approach

It is instructive to examine concentration in tissues and relative infusion rates as both a self-check of reliability and as an indicator of tissue metabolic capacity. Several constants and/or assumptions are useful: 1 mol of H_2S is equivalent to 32 g/L of sulfur, cells are typically 10%–20% protein and 70% water, plasma volume of most mammals is ~4% of body weight (bwt), extracellular fluid volume is 20% of bwt, and total body water is 60% of bwt. A few simple calculations allow the reader (or investigator) to determine the feasibility (and credibility) of reported values.

H_2S (or sulfide) is often measured in tissue or cells in culture and expressed as moles or weight of H_2S or sulfide per gram protein. Assuming cells are 15% protein, 70% water (cytosol), and 1 g of water equals 1 mL, then every gram of cell protein is equivalent to 4.7 mL of water (70/15 = 4.66). Therefore, 1 μmol H_2S/g protein = 1 μmol/4.7 g water. Normalizing to 1 L of cytosol, 1 μmol H_2S/g protein = 214 μM (6.85 mg/L sulfur) in cytosol. When H_2S is reported in micromoles per milligram of protein, 1 μmol of H_2S/mg protein = 214 mM H_2S in cytosol. Tissue H_2S concentrations based on a protein range from 6 nmol/L cytosol in liver (0.03 μmol/kg protein) (37) to tens of millimoles per liter of cytosol (36). Looking at this from a different perspective, if 40 μmol/L H_2S is toxic to cells, this is equivalent to 0.19 μmol/g protein (0.19 nmol/mg protein) or 6.1 μg sulfur/g protein (6.1 ng sulfur/mg protein). Lee *et al.* (36) measured H_2S in cultured U118 cells exposed to a single treatment of 10 μM H_2S and 12 hours later measured 71 μmol/g protein H_2S (Fig. 3). Based on the preceding calculations, this is equivalent to 15,123 μM H_2S in the cytosol. Space does not permit compilation of other values from the literature and the reader is encouraged to evaluate these with the above calculations in mind.

H_2S is also injected as a single bolus or infused over extended periods and the dose is expressed per kilogram of body weight. If 1 μmol/kg bwt H_2S was injected, the H_2S concentration would be 25 μmol/L if it was retained in the plasma, 5 μmol if retained in the extracellular compartment, and 1.7 μmol/L if distributed throughout body water. These values would increase to 1500, 300, and 102 μmol/L if the H_2S was infused for 1 hour and retained within the respective compartment. Bolus injections are typically administered in as small a volume as practicable to minimize volume disturbances and the concentration of H_2S is often quite high; *e.g.*, Wintner *et al.* (77) injected 70 mM Na_2S in 10 seconds. These concentrations are undoubtedly toxic until the H_2S is diluted and metabolized.

H_2S as a signaling molecule

The development of mutant mice lacking CSE ($CSE^{-/-}$) and subsequent observations that plasma H_2S concentration and aortic and heart H_2S production in these mice was less than that of the wild-type mice and that the $CSE^{-/-}$ mutant developed hypertension more frequently compared to the wild type led the authors to conclude that "These findings provide direct evidence H_2S that is a physiologic vasodilator and regulator of blood pressure" (78). However, this conclusion seems a bit premature. First, as already described, plasma H_2S concentrations measured in this study were unrealistically high (40, 32, and 18 μmol/L for wild type, $CSE^{-/+}$, and $CSE^{-/-}$, respectively). Second, Ishii *et al.* (28) also developed $CSE^{-/-}$ mutant mice that exhibited hypercystathioninemia and hyperhomocysteinemia but these mice remained normotensive. Third, the only physiologically relevant stimulus that has thus far been demonstrated to affect H_2S production or concentration in tissues is the inverse correlation between H_2S and Po_2 described above and this mechanism hardly seems applicable in the study by Yang *et al.* (78).

The inverse correlation between Po_2 and H_2S, the similarity of tissue responses to H_2S and hypoxia, and the ability of inhibitors of H_2S synthesis to inhibit hypoxic responses and of sulfur donors to augment it, have led to the hypothesis that H_2S production/metabolism functions as an oxygen sensor in vascular and nonvascular smooth muscle and in chemoreceptor cells (reviewed by Olson and Whitfield [56]). Recent studies of the mammalian carotid body (40, 58, 69, 70) have provided additional support for this mechanism. However, this too has been called into question. Haouzi *et al.* (23)

injected rats with sodium nitrite to produce methemoglobin in blood, which is a well-known scavenger of H_2S. They observed that while intravenous injection of H_2S stimulated ventilation in control rats it did not stimulate ventilation in nitrite-treated rats, thus confirming the H_2S-scavenging effect of methemoglobin They also observed that hypoxic hyperventilation was unaffected by nitrite injection and concluded that H_2S was not involved in H_2S sensing by the carotid body. Key to this conclusion is the ability of methemoglobin to create a sink for H_2S in blood that would prevent buildup of H_2S in the glomus cells of the carotid body. Olson (54) argued against this reasoning based on observations that plasma H_2S is already so low that methemoglobin would not be expected to significantly change the gradient of H_2S between the carotid body and blood, and therefore methemoglobin would not be expected to affect intracellular H_2S signaling. Obviously, this is another conundrum of H_2S biology remaining to be resolved.

It is generally assumed that the metabolic fate of H_2S is mitochondrial oxidation, first to thiosulfate and then sulfate. Thus, once formed, H_2S proceeds inexorably to its fate. However, a recent study by Mikami *et al.* (46) suggested that this might not be always the case. They found that thiosulfate formed from H_2S oxidation in the mitochondria could be reduced by mitochondrial 3-MST or rhodanase in the presence of physiological levels of dihydrolipoic acid (the reduced form of lipoic acid), thereby again forming H_2S. This is an intriguing possibility because it allows recycling of H_2S without consuming additional cysteine or other sulfur-donating biomolecules. This may have special significance in oxygen sensing in which the proposed increase in H_2S as a result of decreased mitochondrial oxidation (56) may be augmented by H_2S production from preformed thiosulfate, especially since the latter would also be expected to be increased by the hypoxia-driven increased reducing conditions in the cell.

Acknowledgments

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References

1. Abe K and Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 16: 1066–1071, 1996.
2. Al-Magableh MR and Hart JL. Mechanism of vasorelaxation and role of endogenous hydrogen sulfide production in mouse aorta. *Naunyn Schmiedebergs Arch Pharmacol* 383: 403–413, 2011.
3. Bagarinao T. Sulfide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms. *Aquat Toxicol* 24: 21–62, 1992.
4. Bearden SE, Beard RS Jr, and Pfau JC. Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress. *Am J Physiol Heart Circ Physiol* 299: H1568–1576, 2010.
5. Caliendo G, Cirino G, Santagada V, and Wallace JL. Synthesis and biological effects of hydrogen sulfide (H_2S): development of H_2S-releasing drugs as pharmaceuticals. *J Med Chem* 53: 6275–6286, 2010.
6. Carballal S, Trujillo M, Cuevasanta E, Bartesaghi S, Moller MN, Folkes LK, Garcia-Bereguiain MA, Gutierrez-Merino C, Wardman P, Denicola A, Radi R, and Alvarez B. Reactivity of hydrogen sulfide with peroxynitrite and other oxidants of biological interest. *Free Radic Biol Med* 50: 196–205, 2011.
7. Chang L, Geng B, Yu F, Zhao J, Jiang H, Du J, and Tang C. Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats. *Amino Acids* 34: 573–585, 2008.
8. Chen KY and Morris JC. Kinetics of oxidation of aqueous sulfide by O_2. *Environ Sci Technol* 6: 529–537, 1972.
9. Chen KY and Morris JC. Oxidation of sulfide by O_2: catalysis and inhibition. *J Sanit Eng Div Amer Soc Civil Eng* 98: 215–227, 1972.
10. Chiku T, Padovani D, Zhu W, Singh S, Vitvitsky V, and Banerjee R. H_2S biogenesis by human cystathionine gamma-lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia. *J Biol Chem* 284: 11601–11612, 2009.
11. Collman JP, Ghosh S, Dey A, and Decreau RA. Using a functional enzyme model to understand the chemistry behind hydrogen sulfide induced hibernation. *Proc Natl Acad Sci U S A* 106: 22090–22095, 2009.
12. DeBruyn WJ, Swartz E, Hu JH, Shorter JA, Davidovits P, Worsnop DR, Zahniser MS, and Kolb CE. Henry's law solubilities and Setchenow coefficients for biogenic reduced sulfur species obtained from gas-liquid uptake measurements. *J Geophys Res* 100: 7245–7251, 1995.
13. DeLeon ER, Stoy GF, and Olson KR. Passive loss of hydrogen sulfide in biological experiments. Anal Biochem. In press, 2011.
14. Doeller JE, Isbell TS, Benavides G, Koenitzer J, Patel H, Patel RP, Lancaster JR, Darley-Usmar VM, and Kraus DW. Polarographic measurement of hydrogen sulfide production and consumption by mammalian tissues. *Anal Biochem* 341: 40–51, 2005.
15. Dombkowski RA, Russell MJ, Schulman AA, Doellman MM, and Olson KR. Vertebrate phylogeny of hydrogen sulfide vasoactivity. *Am J Physiol Regul Integr Comp Physiol* 288: R243–R252, 2005.
16. Eghbal MA, Pennefather PS, and O'Brien PJ. H_2S cytotoxicity mechanism involves reactive oxygen species formation and mitochondrial depolarisation. *Toxicology* 203: 69–76, 2004.
17. Elsey DJ, Fowkes RC, and Baxter GF. Regulation of cardiovascular cell function by hydrogen sulfide (H_2S). *Cell Biochem Funct* 28: 95–106, 2010.
18. Furne J, Saeed A, and Levitt MD. Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am J Physiol Regul Integr Comp Physiol* 295: R1479–R1485, 2008.
19. Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J, and Tang C. H_2S generated by heart in rat and its effects on cardiac function. *Biochem Biophys Res Commun* 313: 362–368, 2004.
20. Gong QH, Wang Q, Pan LL, Liu XH, Huang H, and Zhu YZ. Hydrogen sulfide attenuates lipopolysaccharide-induced cognitive impairment: a pro-inflammatory pathway in rats. *Pharmacol Biochem Behav* 96: 52–58, 2010.
21. Goubern M, Andriamihaja M, Nubel T, Blachier F, and Bouillaud F. Sulfide, the first inorganic substrate for human cells. *FASEB J* 21: 1699–1706, 2007.
22. Guidotti TL. Hydrogen sulfide: advances in understanding human toxicity. *Int J Toxicol* 2010.
23. Haouzi P, Bell H, and Philmon M. Hydrogen sulfide oxidation and the arterial chemoreflex: effect of methemoglobin. *Respir Physiol Neurobiol* 2011.

24. Hu LF, Lu M, Wong PT, and Bian JS. Hydrogen sulfide: neurophysiology and neuropathology. *Antioxid Redox Signal* 15: 405–419, 2011.
25. Hughes MN, Centelles MN, and Moore KP. Making and working with hydrogen sulfide: the chemistry and generation of hydrogen sulfide in vitro and its measurement in vivo: a review. *Free Radic Biol Med* 47: 1346–1353, 2009.
26. Insko MA, Deckwerth TL, Hill P, Toombs CF, and Szabó C. Detection of exhaled hydrogen sulphide gas in rats exposed to intravenous sodium sulphide. *Br J Pharmacol* 157: 944–951, 2009.
27. Ishigami M, Hiraki K, Umemura K, Ogasawara Y, Ishii K, and Kimura H. A source of hydrogen sulfide and a mechanism of its release in the brain. *Antioxid Redox Signal* 11: 205–214, 2009.
28. Ishii I, Akahoshi N, Yamada H, Nakano S, Izumi T, and Suematsu M. Cystathionine gamma-lyase-deficient mice require dietary cysteine to protect against acute lethal myopathy and oxidative injury. *J Biol Chem* 285: 26358–26368, 2010.
29. Jeroschewski P and Steuckart CKM. An amperometric microsensor for the determination of H_2S in aquatic environments. *Anal Chem* 68: 4351–4357, 1996.
30. Kabil O and Banerjee R. Redox biochemistry of hydrogen sulfide. *J Biol Chem* 285: 21903–21907, 2010.
31. Kabil O, Vitvitsky V, Xie P, and Banerjee R. The quantitative significance of the transsulfuration enzymes for H_2S production in murine tissues. *Antioxid Redox Signal* 15: 363–372, 2011.
32. Kasparek MS, Linden DR, Kreis ME, and Sarr MG. Gasotransmitters in the gastrointestinal tract. *Surgery* 143: 455–459, 2008.
33. Kimura H. Hydrogen sulfide: from brain to gut. *Antioxid Redox Signal* 12: 1111–1123, 2010.
34. Kraus DW and Doeller JE. Sulfide consumption by mussel gill mitochondria is not strictly tied to oxygen reduction: measurements using a novel polargraphic sulfide sensor. *J Exp Biol* 207: 3667–3679, 2004.
35. Lagoutte E, Mimoun S, Andriamihaja M, Chaumontet C, Blachier F, and Bouillaud F. Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes. *Biochim Biophys Acta* 1797: 1500–1511, 2010.
36. Lee M, Sparatore A, Del SP, McGeer E, and McGeer PL. Hydrogen sulfide-releasing NSAIDs attenuate neuroinflammation induced by microglial and astrocytic activation. *Glia* 58: 103–113, 2010.
37. Levitt MD, Abdel-Rehim MS, and Furne J. Free and acid-labile hydrogen sulfide concentrations in mouse tissues: anomalously high free hydrogen sulfide in aortic tissue. *Antioxid Redox Signal* 15: 373–378, 2011.
38. Li L, Rose P, and Moore PK. Hydrogen sulfide and cell signaling. *Annu Rev Pharmacol Toxicol* 51: 169–187, 2011.
39. Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, Zhao Y, Baskar R, Tan CH, and Moore PK. Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation* 117: 2351–2360, 2008.
40. Li Q, Sun B, Wang X, Jin Z, Zhou Y, Dong L, Jiang LH, and Rong W. A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. *Antioxid Redox Signal* 12: 1179–1189, 2010.
41. Li YF, Xiao CS, and Hui RT. Calcium sulfide (CaS), a donor of hydrogen sulfide (H_2S): a new antihypertensive drug? *Med Hypotheses* 73: 445–447, 2009.
42. Linden DR, Levitt MD, Farrugia G, and Szurszewski JH. Endogenous production of H_2S in the gastrointestinal tract: still in search of a physiologic function. *Antioxid Redox Signal* 12: 1135–1146, 2010.
43. Lippert AR, New EJ, and Chang CJ. Reaction-based fluorescent probes for selective imaging of hydrogen sulfide in living cells. *J Am Chem Soc* 133: 10078–10080, 2011.
44. Mancardi D, Penna C, Merlino A, Del SP, Wink DA, and Pagliaro P. Physiological and pharmacological features of the novel gasotransmitter: hydrogen sulfide. *Biochim Biophys Acta* 1787: 864–872, 2009.
45. Martelli A, Testai L, Breschi MC, Blandizzi C, Virdis A, Taddei S, and Calderone V. Hydrogen sulphide: novel opportunity for drug discovery. *Med Res Rev* 2010 [Epub ahead of print].
46. Mikami Y, Shibuya N, Kimura Y, Nagahara N, Ogasawara Y, and Kimura H. Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochem J* 439, 479–485, 2011.
47. Muzaffar S, Jeremy JY, Sparatore A, Del SP, Angelini GD, and Shukla N. H_2S-donating sildenafil (ACS6) inhibits superoxide formation and gp91phox expression in arterial endothelial cells: role of protein kinases A, and G. *Br J Pharmacol* 155: 984–994, 2008.
48. Nagy P and Winterbourn CC. Rapid reaction of hydrogen sulfide with the neutrophil oxidant hypochlorous acid to generate polysulfides. *Chem Res Toxicol* 2010.
49. Nicholson CK and Calvert JW. Hydrogen sulfide and ischemia-reperfusion injury. *Pharmacol Res* 62: 289–297, 2010.
50. Nikolaidis MG and Jamurtas AZ. Blood as a reactive species generator and redox status regulator during exercise. *Arch Biochem Biophys* 490: 77–84, 2009.
51. Norris EJ, Culberson CR, Narasimhan S, and Clemens MG. The liver as a central regulator of hydrogen sulfide. *Shock* 36: 242–250, 2011.
52. Olson KR. Vascular actions of hydrogen sulfide in non-mammalian vertebrates. *Antioxid Redox Signal* 7: 804–812, 2005.
53. Olson KR. Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? *Biochim Biophys Acta* 1787: 856–863, 2009.
54. Olson KR. "Hydrogen sulfide oxidation and the arterial chemoreflex: effect of methemoglobin" by P. Haouzi *et al.* [Respir. Physiol. Neurobiol. (2011), doi:10.1016/j.resp.2011.04.025]. *Respir Physiol Neurobiol* 179: 121, 2011.
55. Olson KR. The therapeutic potential of hydrogen sulfide: separating hype from hope. *Am J Physiol Regul Integr Comp Physiol* 301: R297–R312, 2011.
56. Olson KR and Whitfield NL. Hydrogen sulfide and oxygen sensing in the cardiovascular system. *Antioxid Redox Signal* 12: 1219–1234, 2010.
57. Olson KR, Whitfield NL, Bearden SE, St. Leger J., Nilson E, Gao Y, and Madden JA. Hypoxic pulmonary vasodilation: A paradigm shift with a hydrogen sulfide mechanism. *Am J Physiol Regul Integr Comp Physiol* 298: R51–R60, 2010.
58. Peng YJ, Nanduri J, Raghuraman G, Souvannakitti D, Gadalla MM, Kumar GK, Snyder SH, and Prabhakar NR. H_2S mediates O_2 sensing in the carotid body. *Proc Natl Acad Sci U S A* 107: 10719–10724, 2010.
59. Predmore BL and Lefer DJ. Development of hydrogen sulfide-based therapeutics for cardiovascular disease. *J Cardiovasc Transl Res* 3: 487–498, 2010.

60. Qian Y, Karpus J, Kabil O, Zhang S-Y, Zhu H-L, Banerjee R, Zhao J, and He C. Selective fluorescent probes for live-cell monitoring of sulphide. *Nat Commun* doi: 10.1038/ncomms1506: 2011.
61. Searcy DG and Lee SH. Sulfur reduction by human erythrocytes. *J Exp Zool* 282: 310–322, 1998.
62. Sen U, Mishra PK, Tyagi N, and Tyagi SC. Homocysteine to hydrogen sulfide or hypertension. *Cell Biochem Biophys* 57: 49–58, 2010.
63. Shen X, Pattillo CB, Pardue S, Bir SC, Wang R, and Kevil CG. Measurement of plasma hydrogen sulfide in vivo and in vitro. *Free Radic Biol Med* 50: 1021–1031, 2011.
64. Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, and Kimura H. 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal* 11: 703–714, 2009.
65. Singh S, Padovani D, Leslie RA, Chiku T, and Banerjee R. Relative contributions of cystathionine beta-synthase and gamma-cystathionase to H_2S biogenesis via alternative trans-sulfuration reactions. *J Biol Chem* 284: 22457–22466, 2009.
66. Stipanuk MH and Ueki I. Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. *J Inherit Metab Dis* 34: 17–32, 2010.
67. Szabó C. Hydrogen sulphide and its therapeutic potential. *Nat Rev Drug Discov* 6: 917–935, 2007.
68. Szabó G, Veres G, Radovits T, Gero D, Módis K, Miesel-Gröschel C, Horkay F, Karck M, and Szabó C. Cardioprotective effects of hydrogen sulfide. *Nitric Oxide* 25: 201–210, 2010.
69. Telezhkin V, Brazier SP, Cayzac S, Muller CT, Riccardi D, and Kemp PJ. Hydrogen sulfide inhibits human BK(Ca) channels. *Adv Exp Med Biol* 648: 65–72, 2009.
70. Telezhkin V, Brazier SP, Cayzac SH, Wilkinson WJ, Riccardi D, and Kemp PJ. Mechanism of inhibition by hydrogen sulfide of native and recombinant BKCa channels. *Respir Physiol Neurobiol* 172: 169–178, 2010.
71. Toombs CF, Insko MA, Wintner EA, Deckwerth TL, Usansky H, Jamil K, Goldstein B, Cooreman M, and Szabó C. Detection of exhaled hydrogen sulphide gas in healthy human volunteers during intravenous administration of sodium sulphide. *Br J Clin Pharmacol* 69: 626–636, 2010.
72. Ubuka T. Assay methods and biological roles of labile sulfur in animal tissues. *J Chromatogr B Analyt Technol Biomed Life Sci* 781: 227–249, 2002.
73. Ubuka T, Abe T, Kajikawa R, and Morino K. Determination of hydrogen sulfide and acid-labile sulfur in animal tissues by gas chromatography and ion chromatography. *J Chromatogr B Biomed Sci Appl* 757: 31–37, 2001.
74. Wang R. Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? *FASEB J* 16: 1792–1798, 2002.
75. Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong BS, Cheung NS, Halliwell B, and Moore PK. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *J Neurochem* 90: 765–768, 2004.
76. Whitfield NL, Kreimier EL, Verdial FC, Skovgaard N, and Olson KR. A reappraisal of H_2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. *Am J Physiol Regul Integr Comp Physiol* 294: R1930–R1937, 2008.
77. Wintner EA, Deckwerth TL, Langston W, Bengtsson A, Leviten D, Hill P, Insko MA, Dumpit R, VandenEkart E, Toombs CF, and Szabó C. A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. *Br J Pharmacol* 160: 941–957, 2010.
78. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, and Wang R. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322: 587–590, 2008.
79. Zhang H and Bhatia M. Hydrogen sulfide: a novel mediator of leukocyte activation. *Immunopharmacol Immunotoxicol* 30: 631–645, 2008.
80. Zuidema MY, Yang Y, Wang M, Kalogeris TJ, Liu Y, Meininger CJ, Hill MA, Davis MJ, and Korthuis RJ. Antecedent hydrogen sulfide elicits an anti-inflammatory phenotype in postischemic murine small intestine: role of BK channels. *Am J Physiol Heart Circ Physiol* 2010.

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Abbreviations Used

CBS = cystathionine β-synthase
CSE = cystathionine γ-lyase
$CSE^{E/E}$ = mice lacking cystathionine γ-lyase
DTPA = diethylenetriaminepentaacetic acid
GYY4137 = morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC = high pressure liquid chromatography
H_2S = hydrogen sulfide
IK-1001 = sterile Na_2S solution
ISE = ion-selective electrodes
k = rate constant
MBB = monobromobimane
3-MST = 3-mercaptopyruvate sulfur transferase
NO = nitric oxide
Po_2 = oxygen partial pressure
ppb = parts per billion
ppm = parts per million
ROS = reactive oxygen species
RSnH = persulfide
RS-S = sulfane sulfur
$t_{½}$ = half time

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