H₂S concentrations in the arterial blood during H₂S administration in relation to its toxicity and effects on breathing

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During H₂S exposure, a large portion of H₂S diffusing into the blood is oxidized into innocuous compounds (sulfite, sulfate, and thiosulfate) (8, 16, 22). This oxidation takes place both in the blood and in most tissues (cytoplasm and mitochondria) (for review, see Ref. 37), limiting, in turn, the dreadful effects of H₂S toxicity. However, even at very low levels of exogenous H₂S exposure, not all H₂S is “oxidized”. H₂S appears in the arterial blood (and thus by diffusion, increases in the tissue) during inhalation as low as 50 ppm or during venous infusion levels of H₂S as low as 1 μmol/min in rats and humans (8, 29, 46, 54).

However, H₂S can be transported in the blood in different forms, i.e., dissolved and combined. These forms are not equivalent in terms of their physiological effects. The dissolved H₂S consists of 1) H₂S in gaseous form with a concentration proportional to its partial pressure (PH₂S), according to Henry’s law (3, 6, 10, 13, 17), and 2) the sulphydryl anion, HS⁻ (3, 34). The gaseous form of H₂S is, at physiological pH, about 20–30% of the total dissolved H₂S (27). H₂S and HS⁻ represent the only forms under which hydrogen sulfide can diffuse between blood and tissues or within cells. On the other hand, H₂S can be combined with 1) metalloproteins (e.g., hemoglobin), sometimes referred to as acid-labile sulfides (7, 42, 43), and 2) cysteine residues leading to the formation of disulfide bonds (R-S-S-H), also referred to as persulfides or sulfhydrated proteins (35, 38, 51). From a physiological standpoint, the combined forms play a dual role since they represent a mechanism of protection (41, 43, 47), trapping H₂S in a non-soluble form, but also account for some of the effects of H₂S toxicity (e.g., interaction with cytochrome-c oxidase) (12, 16, 31, 33, 41). Similarly, the alteration of specific enzymatic activities by the process of sulfhydration offers novel pathways through which both exogenous and endogenous H₂S could exert their toxic and physiological effects, respectively (38).

Despite decades of research on the fate of H₂S in the blood (22), there is, as yet, no direct information on the relative contribution of the different forms of H₂S transport and on the relationship between H₂S concentrations and the acute clinical/toxic manifestations produced by sulfide during and following H₂S exposure (8, 21, 41). As a consequence, any attempt to rationalize the use of potential antidotes, e.g., methemoglobin, hydroxocobalamin, bicarbonate, O₂, or reducing agents (21, 41, 44, 51), remains speculative. In addition, H₂S has long been shown to exert its main toxicity by inhibiting the activity of mitochondrial cytochrome-c oxidase (CCO) (12, 31) at concentrations between 10 and 50 μM in vitro. However, we do not know how much soluble/diffusible H₂S must be present in vivo (16) to produce a reduction in CCO activity vs. other mechanisms that account for the main acute, clinical manifestations (41) of sulfide poisoning, such as hyperventilation (25, 28), apnea (1, 20), or coma (2).

In the current study, following experiments from Insko et al. (29) and Wintner et al. (54), we infused H₂S intravenously in the form of NaHS to spontaneously breathing, urethane-anesthetized rats. We increased the infusion rate gradually until an apnea occurred (“lethal” exposure). At each step, the rate of H₂S elimination and the level of dissolved H₂S in the blood were measured along with breathing, used here as a clinical sign of toxicity. We determined the partial pressure of H₂S in the arterial blood, and, thus, its concentration, from the measurement of the alveolar partial pressure of H₂S. In addition, the concentration of H₂S was measured in the arterial blood at specific time points, in steady-state conditions, using the HPLC-fluorescence technique based on complexation of reac-
tive sulfide species with monobromobimane (MBB) (54). The chemical derivatization protocol that we used has been validated elegantly by Wintner et al. (54) as a method to measure sulfide in the blood, not only in its soluble form, but also combined with the red blood cells.

After characterizing the factors potentially affecting the measurement of H2S concentrations using the MBB technique, as well as alveolar H2S partial pressure, we present a quantitative description of the fate of the dissolved and combined forms of H2S in the blood and the limits of the approaches that we have used.

These results are discussed in the light of the potential benefits of putative antidotes against H2S toxicity and the debate over the levels of endogenous H2S required to affect structures involved in breathing control in vivo or in vitro.

METHODS

Animal Preparation

The experimental procedures were performed on 10 adult male, Sprague-Dawley rats (563 ± 136 g), as previously described (26). All procedures were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Anesthesia was induced with 3.5% isoflurane in O2 followed by an intraperitoneal injection of 1.2 g/kg of urethane. A tracheostomy was performed, and a catheter (14 gauge, 2.25 mm OD) was placed in the trachea. The catheter was attached to a Hans Rudolph low dead space two-way valve. The inspiratory port of the valve was connected to a pneumotachograph (1100 Series; Hans Rudolph, KS). Inspiratory flow was measured breath-by-breath. The expiratory port of the valve was connected to two 5-ml “mixing chambers” placed in series. The outlet of the second chamber was connected to a filter containing charcoal. Mixed expired CO2 and H2S fractions were measured continuously from the second mixing chamber, using a CO2 infrared (Vacumed 17630; Vacumed, Ventura, CA) and H2S (Interscan RM series; Interscan, Simi Valley, CA) analyzer, respectively. The range of the H2S analyzer is 0.001 to 1.00 ppm; at the level of infused H2S concentration, the expiration of H2S fraction (FEH2S) was computed as FEH2S = FcH2S × (Ve + Vadd/Vt), where Ve was the alveolar ventilation.

The partial pressure of expired H2S (PEH2S) was then calculated as PEH2S = PB (mmHg).

As the diffusion of H2S is, by definition, only taking place in alveolar regions and not in the pulmonary dead space, the alveolar partial pressure of H2S (PAH2S) was computed as PAH2S = PEH2S × Ve/Vl, where Ve was the alveolar ventilation.

PECO2/PACO2 was determined from arterial PCO2 (PACO2) and arterial CO2 content (CA) sampled at various times during the experiment, while PEH2S was continuously measured from the second mixing chamber.

H2S was equated to PAH2S (neglecting the possibility of pulmonary or extra-pulmonary shunts). The concentration of gaseous H2S in the blood (CgH2S) was calculated as

\[
CgH2S = 0.00012 \times PaH2S, \quad \text{with} \quad 0.00012 \quad \text{being the coefficient of solubility of H2S}.
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PEH2S was estimated from arterial CO2 (PAH2S) sampled at various times during the experiment, while PEH2S was continuously measured from the second mixing chamber.

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Measurements and Data Analysis

The pneumotachograph and blood pressure transducers were calibrated prior to every experiment. The gas analyzers were calibrated using different gas mixtures containing 0 or 5% CO2 for the CO2 analyzer and 0 (air flowing through a charcoal filter) or 0.65 ppm of H2S.

Inspiratory flow, the additional flow of gas delivered (Vadd) to the first mixing chamber, ABP, CO2, and H2S fractions in the second mixing chamber, as well as the rectal temperature signals, were digitized using an analog-to-digital data acquisition system (PowerLab 16/35; AD Instruments; Colorado Springs, CO) at 200 Hz. Data were displayed online and stored for later analysis. Breathing frequency (f) and tidal volume (Vt) were determined using peak detection and integration of the inspiratory flow signal, respectively, and minute ventilation (Vt) was computed as f × Vt.

Alveolar H2S Fraction, Partial Pressure, and Dissolved Concentrations of H2S in the Arterial Blood

The fraction of H2S was continuously measured from the potential mixing chamber, defined as FcH2S. Assuming Ve = Vt, the mixed expired H2S fraction (FEH2S) was computed as FEH2S = FcH2S × (Ve + Vadd/Vt). The partial pressure of expired H2S (PEH2S) was then calculated as PEH2S = PB (mmHg).

As the diffusion of H2S is, by definition, only taking place in alveolar regions and not in the pulmonary dead space, the alveolar partial pressure of H2S (PAH2S) was computed as PAH2S = PEH2S × Ve/Vl, where Ve was the alveolar ventilation.

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standard curves constructed for these analyses. The detection limit was 0.5 ng/injection. An example of a HPLC/fluorescence spectrum is shown in Fig. 1.

See APPENDIX for information regarding reagents and preparation of internal standards.

In Vitro Experiments

In addition to the validation of the MBB technique by Wintner et al. (54), who showed that the CMBBH₂S reflects the concentration of soluble H₂S and H₂S combined to hemoglobin in the blood (see DISCUSSION), we sought to determine whether H₂S present in the blood also interacts with the plasma proteins, or is primarily interacting with red cells. We measured CMBBH₂S in a NaHS solution (final concentration 100 μM) mixed in 0.9% saline (n = 9), fresh rat blood collected from naïve, untreated rats (n = 8), fresh plasma (n = 10), or a solution of Methb ([Hb]=10.0 g/100 ml, >90% Methb) (n = 4). CMBBH₂S was also measured in the plasma with and without the reducing agent Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Soltec Bio Science, Beverly, MA). The rationale was that in the presence of the reducing agent TCEP, which should limit the sulfhydryl-destruction of proteins, one would expect CMBBH₂S to be higher with TCEP than in control plasma. When added to the blood containing TCEP, H₂S concentration should also be higher than in control blood, if the plasma proteins are interacting with H₂S in the whole blood. Finally, in the presence of methemoglobin, we anticipate H₂S and H₂S combined to hemoglobin (see RESULTS). Second, the influence of the additional flow of a gas (Vadd) on the measurement of FH₂S was determined using a gas mixture containing H₂S (0.65 ppm) in N₂. This gas was delivered to the first mixing chamber at a flow ranging from 0 to 22 l/min, while FH₂S was determined from the second mixing chamber.

Validation of FAH₂S measurement. First, the effects of the contact time between H₂S and the gas coming from the alveolar region to the analyzer and the composition of the expired gas were characterized (Fig. 2, A and B). This was achieved by interposing tubing of two different lengths, i.e., 2 m (100 ml, short circuit) or 6.5 m (325 ml, long circuit) corresponding to 0.5 s and 1.4 s, respectively, between the first and second mixing chamber and mixing a gas containing H₂S with an original concentration of 25 ppm diluted with N₂, 100% O₂, or air (see RESULTS). Second, the influence of the additional flow of a gas (Vadd) on the measurement of FH₂S was determined.

All data are expressed as means ± SD. The relationship between alveolar H₂S and H₂S in the blood was established in keeping with the dose infused and the level of minute ventilation. Exponential regression analysis was used to describe the relationship between CH₂S and the rate of H₂S infusion. In addition, a Mann-Whitney rank sum test compared baseline values to measurements taken during the different infusion rates of NaHS. Post hoc comparisons were performed using an unpaired t-test (SigmaStat 2.0; USA). The concentration of H₂S at which breathing was increased by 30% was chosen as the threshold for CB stimulation (H₂S-induced respiratory symptoms).

FH₂S at two different contact times was compared using a paired t-test, and the values of CH₂S and CMBBH₂S at the different rates of infusion were compared using one-way ANOVA. In all instances, P < 0.05 was considered as significant.

RESULTS

In vitro determination of CMBBH₂S in plasma and whole blood. In contrast to saline, in which CMBBH₂S remained unchanged over time, CMBBH₂S decreased dramatically in the plasma reaching 46 ± 3% of CMBBH₂S in saline at 10 min. CMBBH₂S also decreased over time in the whole blood, reaching 29 ± 18% of CMBBH₂S in saline. As shown in Fig. 1, adding 1 mM of the reducing agent TCEP prevented the decrease in CMBBH₂S in plasma (74 ± 16%; P < 0.01) but not in whole blood (21 ± 20%). Finally, CMBBH₂S was found...
to be significantly higher after MetHb than in the blood (59 ± 0.1%; P < 0.01).

Determination of H$_2$S in the Expired Gas

Effect of the time of contact between H$_2$S and the expiratory gas and effect of gas composition. We found no difference between the concentrations of the H$_2$S in air, N$_2$, or 100% O$_2$ or between the short (2 m) or long (6.5 m) circuit (see METHODS section) as shown in Fig. 2. In other words, the time of contact, as well as the composition of the expired gas, had no effect on the fraction of H$_2$S coming from the alveolar region up to 1.5 s. The latter is longer than the time required for a given aliquot of expired gas to reach the analyzer using a sampling flow of about 500 ml/min.

Effect of Gas Flow

To evaluate the influence of $V_{add}$ on the determination of FH$_2$S in the mixing chamber where the expired gas was sampled by our H$_2$S analyzer, the effects of the flow of a gas with a known concentration of H$_2$S in N$_2$ was evaluated in keeping with the levels of $V_{add}$ that we used in vivo (Fig. 2). FH$_2$S increased by less than 6% between 0 and 22 l/min, likely due to the change in pressure in the mixing chamber at a high flow.

CgH$_2$S and CMBBH$_2$S During H$_2$S Infusion: Effects on Breathing

Figure 3 is an example of the change in the alveolar fraction of H$_2$S at various rates of NaHS infusion along with ventilation. H$_2$S was undetectable in its soluble form in baseline conditions, while CMBBH$_2$S averaged 0.7 ± 0.5 μM. Clearly, CgH$_2$S increased according to the level of H$_2$S infused, and CMBBH$_2$S followed a very similar pattern, but at a level that was about 50 times that of CgH$_2$S at low concentrations and 5 or 6 times that of the lethal level. The average values of CgH$_2$S and CMBBH$_2$S, as a function of the rate of H$_2$S infusion, are displayed in Figs. 4 and 5.

CgH$_2$S was found to average 1.1 ± 0.7 μM when breathing was stimulated (H$_2$S partial pressure of 9.5 $\times$ 10$^{-3}$ ± 5.5 $\times$ 10$^{-3}$ mmHg), corresponding to a CMBBH$_2$S of 11.1 ± 5.4 μM. At lower concentrations, no visible effect on breathing was observed, as illustrated in Fig. 3. The change in breathing occurred as soon as the rate of infusion was increased to 6.10 μmol/min. Minute ventilation increased significantly from 284 ± 6 ml/min to 383 ± 8 ml/min (P < 0.01). This phase of hyperventilation was associated with the production of numerous augmented breaths, along with phases of periodic breathing typical of carotid body stimulation. Depression of breathing, leading to apnea, occurred between 5.1 and 8.8 μM for CgH$_2$S and 25.4 and 50.9 μM for CMBBH$_2$S, as illustrated in Figs. 3 and 5.

The rate of infusion, which produced a terminal apnea (8.37–10.46 μmol/min), was only 37% higher than the rate at which NaHS increased breathing. This small rise in the NaHS infusion rate increased the concentration of gaseous H$_2$S by about 8 times, while CMBBH$_2$S rose by 4.6 times. Typically, the apneic response triggered by NaHS consisted of a rapid depression in breathing frequency for about 1 min followed by a complete cessation of breathing. In a few tests, the period of depression was limited to about 10 s, leading to an apnea. Apneic rats were mechanically ventilated, and all rats but two resumed a spontaneous breathing pattern within 30–60 s after the cessation of NaHS infusion. In five of these animals, CH$_2$S was determined during recovery following a new infusion of a sublethal level of NaHS at an average rate of 4.42 μmol/min for 4 min (See METHODS section). We had previously measured the time constant of our H$_2$S analyzer and the respiratory circuit and found it to be between 10 and 12 s at a sampling rate of 500 ml/min. As soon as NaHS infusion was interrupted, CgH$_2$S dropped dramatically. The kinetics of this response was indiscernible from the off-transient of the analyzer. CMBBH$_2$S...
subsided progressively over time and remained above baseline, even after 15 min (Fig. 6).

**Balance of H₂S**

The rate of H₂S eliminated per minute by the lungs averaged 0.0058 ± 0.0023 μmol/min for the lowest rate of infusion (2.09 μmol/min), corresponding, therefore, to 0.28% of the rate of H₂S infused. The rate of elimination by the lungs reached 0.57 ± 0.36 μmol/min during infusion of lethal levels of H₂S (about 5.4% of 10.46 μmol/min). For all of the rates of infusion, except the one leading to apnea, FH₂S remained stable during infusion, suggesting, therefore, that the rate of elimination of H₂S (e.g., via oxidation, or combination to proteins) was about the same as the rate of infusion.

**DISCUSSION**

We found that in the rat, the concentrations of both dissolved and combined H₂S displayed an exponential relationship with the rate/concentration of H₂S infused in the venous blood, until breathing was abolished (lethal dose). CgH₂S represents a small portion of the total concentration of H₂S present in the arterial blood. CMBBH₂S/CgH₂S ratio ranged from 50 for the lowest concentrations to 6 when lethal levels were reached. When the first clinical signs of H₂S exposure occurred, i.e., breathing stimulation, H₂S averaged 1.14 M for CgH₂S and 11.1 M for CMBBH₂S. H₂S produced a terminal apnea, and was thus lethal, at concentrations of gaseous and “combined” H₂S, which ranged from 5.1 to 9.8 μM and from 27.1 to 50.8 μM.
µM, respectively. These results complement the data previously published on the concentrations of H₂S in the blood using the MBB technique in the rat (54) and also offer a simple way to estimate the concentrations of dissolved H₂S in the blood based on the continuous determination of alveolar H₂S partial pressure. Collectively, these data allow us to propose a frame of reference that can be used to study and clarify the clinical relevance of studies using exogenous sulfide to mimic the effects of endogenous H₂S, as well as the potential impacts of antidotes against H₂S intoxication.

What Was Measured in the Blood?

Wintner et al. (54) have already used and validated the approach based on the formation of sulfide-dibimane resulting from the reaction of H₂S and MBB to determine the change in H₂S concentrations in the blood during H₂S inhalation or intravenous infusion. Sulfide-dibimane was identified, and its concentration was determined by reverse-phase HPLC separation coupled to fluorescence detection. Because we used a different and larger range of H₂S concentrations, not all of our data could be compared with those reported by Wintner et al. (54), who studied low levels of exposure. We found, however, values very similar to those reported in that low range (See Fig. 5). We also found that CMBBH₂S increased sharply, with a very steep slope, when the rate of H₂S administration was close to the lethal levels of exposure, corresponding to a CMBBH₂S around 30 – 40 µM. Wintner et al. (54) also showed, using a series of in vitro studies, that the MBB technique can identify H₂S present in various forms in the blood. First, H₂S concentrations in the plasma were similar to the values obtained using amperometry, which only measures soluble H₂S (15, 30, 37). We found that in saline, CMBBH₂S did not change within 10 min, if evaporation of H₂S was prevented, in keeping with our previous results (49, 50). In addition, since H₂S is present in the form of HS⁻ (70 – 80%) and H₂S (30 – 20%) at physiological pH in PBS, CMBBH₂S includes all forms of dissolved H₂S (gaseous and sulfhydryl anion HS⁻).

Perhaps more importantly, these authors reported that when exogenous H₂S was added to whole blood, it disappeared virtually within seconds in its soluble form, as determined by amperometry, which only measures soluble H₂S (15, 30, 37). We found that in saline, CMBBH₂S did not change within 10 min, if evaporation of H₂S was prevented, in keeping with our previous results (49, 50). In addition, since H₂S is present in the form of HS⁻ (70 – 80%) and H₂S (30 – 20%) at physiological pH in PBS, CMBBH₂S includes all forms of dissolved H₂S (gaseous and sulfhydryl anion HS⁻).

Fig. 5. A: CgH₂S as a function of the rate of H₂S infused (only mean values are shown). Breathing was stimulated at a concentration of CgH₂S around 1.1 µM, while the highest values corresponding to the lethal level ranged from 5.1 to 8.8 µM. The data recomputed from the study of Insko et al. (diamonds) fits with the relationship established in the present study (29). B: CMBBH₂S as a function of the rate of H₂S infused compared with data from Wintner et al. (triangles) (54). C: relationship between mean CgH₂S and mean CMBBH₂S. D: relationship between the CMBBH₂S/CgH₂S ratio and CgH₂S. Note that this ratio is very high and decreases in keeping with the rate of H₂S infused.

Fig. 6. CMBBH₂S during recovery from NaHS infusion. CMBBH₂S was still significantly higher than baseline 15 min into recovery from H₂S exposure. **P < 0.01.
for the determination of H$_2$S in its dissolved form but also measures sulfide combined, in part, with Hb, 2) the MBB technique does not measure H$_2$S sulfhydrated on the cysteine residues of protein in the plasma, 3) H$_2$S is primarily interacting with hemoglobin when entering the blood, preventing further reaction with the proteins in the plasma, and 4) the progressive decrease in H$_2$S in the whole blood or in the MetHb solution in plasma reflects its oxidation by the ferrous/ferric iron.

The concentrations of dissolved H$_2$S in the arterial blood in vivo can be measured by the amperometry technique (15, 30), which requires careful and repetitive calibrations of the electrode. As pointed out in the previous paragraph, the concentrations of H$_2$S reported by amperometry greatly differ in vitro from CMBBH$_2$S in the blood (54), but not in the plasma or in saline. This is also true in vivo: for instance, in Fig. 11 of their paper, Wintner et al. (54) showed that the concentrations of dissolved H$_2$S in the blood barely reach 1 $\mu$M, while CMBBH$_2$S averages 30 $\mu$M during sodium sulfide infusion at a rate of 1 mg·kg$^{-1}$·min$^{-1}$. This ratio between dissolved H$_2$S and CMBBH$_2$S is of the same magnitude as the ratio between dissolved and combined H$_2$S we found in our study, while using the partial pressure of H$_2$S in the alveolar gas. This ratio decreased as the concentration or rate of H$_2$S infusion reached lethal levels. We determined the arterial partial pressure of H$_2$S in the blood from the measurement of mixed expired H$_2$S and the ratio between mixed expired and arterial PCO$_2$ (or V̇E/V̇A), allowing the estimation of the concentration of dissolved H$_2$S in the arterial blood in gaseous form. Indeed, when the H$_2$S rate of disappearance equals its rate of infusion, H$_2$S should be in equilibrium in all body compartments. A plateau of expired H$_2$S values was reached for all the sublethal levels of exposure to H$_2$S, so PH$_2$S is expected to be similar in the alveolar gas and in the blood leaving the lungs. When reaching values close to the levels producing apnea, no steady state could be reached (Fig. 5) and PH$_2$S was probably no longer in equilibrium in the lungs and the blood. This approach, which differs from traditional amperometry, relies on a certain number of prerequisites and limits. We found that the RM17-1000b hydrogen sulfide detector, which was previously used to estimate the rate of elimination of H$_2$S by the lungs (29), is sensitive to change in humidity, which would require it to re-zero when connected to expired gas unless dry gas is used. Second, the sampling flow of these analyzers is high (around 0.5 l/min, which is about 2 times the minute ventilation of a rat) but does fluctuate around its set value. As developed in the METHODS, we used an additional flow of dry gas, which overcame the possible variable dilution related to this nonstable flow and, more importantly, the additional flow allowed us to use the same analyzer in a much higher range and avoided the effect of humidity. Yet, the FAH$_2$S values that we are reporting are very close to those recomputed from the study of Insko et al. (29)—with the same analyzer—using the values of mixed, expired H$_2$S, and assuming a $V_A/V_e$ ratio of 0.7.

We selected a Henry’s (H0) coefficient of 0.084 M·l$^{-1}$·atm$^{-1}$ for a temperature of 36°C. The solubility (H) of H$_2$S was computed using a value of H0 determined from the studies of Douabal and Riley. (17) and Barrett et al. (6) after correction with a Satchenow coefficient of 0.064 (13) [$H = H0e^{\text{-0.064}M/H0}$]. This yielded values similar to the actual data reported in a saline solution by Douabal et al. (17). Also, at a pH of 6.9, at least 70 to 80% or more of H$_2$S is present under the HS$^-$ form (3, 34), so that the total concentration of dissolved hydrogen sulfide can be assumed to be about 3 times CgH$_2$S.

Even if we assume that H$_2$S was in equilibrium in all body “water compartments” and taking into account the rate of pulmonary elimination (see RESULTS), from only 1 to 5% of the anticipated concentrations of H$_2$S was found in the blood in the form of CgH$_2$S. Five to 20% of the expected H$_2$S concentrations were identified by MBB. Clearly, the majority of H$_2$S may have been rapidly oxidized in the blood and in the tissues (32). The possibility that some of the H$_2$S infused could have been combined with compounds “invisible” to the MBB technique should be also tested.

**Baseline H$_2$S and Recovery From H$_2$S Exposure**

Haggard (22), in his seminal paper on “the fate of H$_2$S in the blood,” was among the first to report that as soon as H$_2$S diffuses in the blood, it virtually disappears. Furne et al. (19) convincingly showed that in baseline conditions H$_2$S could only be present in the blood at best in the micromolar range. Whitfield et al. (52) also challenged the numerous studies wherein the presence of endogenous H$_2$S was found to be in the micromolar range in the blood in many species, including in humans, as well as the use of a micromolar concentration of H$_2$S to mimic physiological changes produced by endogenous H$_2$S (for review, see Ref. 36).

We found no measurable level of expired H$_2$S in baseline conditions, while baseline CMBBH$_2$S ranged between 0.3 and 0.8 $\mu$M; similar results have been reported by Wintner et al. (54) in rats and by Tokuda et al. (45) in mice. Baseline CMBBH$_2$S origin and the exact nature remain to be determined: whether CMBBH$_2$S is a marker or can be used as a surrogate of the presence of endogenous H$_2$S, or other thiol compounds, is unknown.

Finally, it is quite interesting that CMBBH$_2$S remains elevated in the blood for at least 15 min after the cessation of H$_2$S exposure. This suggests that H$_2$S may remain combined with metalloprotein compounds in the tissue (such as cytochrome-c oxidase) well after the end of exposure and could account for the beneficial effects of antidotes administered following H$_2$S intoxication in humans (24).

**Concentrations of H$_2$S and Breathing**

The levels of H$_2$S partial pressure, and, therefore, dissolved H$_2$S concentrations, present in the blood were determined in keeping with the clinical respiratory symptoms of H$_2$S toxicity (41). Since the very first description of the effects of exogenous H$_2$S on breathing (23, 28), it has been well established that 1) the ventilatory stimulation produced by H$_2$S is exclusively mediated by the arterial chemoreceptors (28, 53), and 2) H$_2$S-induced apnea is the result of the direct effects of H$_2$S on medullary respiratory neurons (20) and to some extent the stimulation of pulmonary vagal afferents (1), although this latter notion has been challenged. Indeed, in the sheep, the apnic response to H$_2$S is unaltered after bilateral vagotomy (25). Typically, hyperventilation occurs with H$_2$S around 500 ppm in humans, while death occurs by apnea (inaccurately termed “respiratory paralysis”) around 1,000 ppm in the course of an extremely short period of exposure (4, 8, 9, 18, 21, 41). Although these figures should be regarded more as a general
frame of reference rather than absolute thresholds for toxicity, we found that the concentrations of dissolved H$_2$S required to produce these effects were very reproducible between animals. They range within less than 1 $\mu$M for breathing stimulation and less than 8 $\mu$M for the cessation of breathing, i.e., 3 and 24 $\mu$M if total dissolved H$_2$S and HS$^-$ are considered. These concentrations should be compared with the much higher levels of H$_2$S used to stimulate CB in vitro, i.e., between 30 and 100 $\mu$M (39) or the 300 $\mu$M of H$_2$S required to affect breathing control when applied to a brain stem preparation (11). Our present results suggest, that H$_2$S partial pressures and, thus, H$_2$S concentrations required to stimulate breathing in vivo can be quite low, i.e., low micromolar range, in the blood and are, thus, probably even lower in the tissue. This offers the possibility, at least for the stimulatory response, that H$_2$S can act through nonmitochondrial mechanisms. The other possibility is that, in vivo, much lower concentrations of H$_2$S than those reported from in vitro experiments, i.e., using cells, tissue, or mitochondria, are already impeding cytochrome-c oxidase activity in the CB (12, 31).

Clinical Relevance

H$_2$S is traditionally regarded as a chemical hazard in oil and gas production (5, 18) and in well drilling and gas refining (9, 18). Because H$_2$S can be easily manufactured, using material readily available to everyone, it has sadly become an appalling method of suicide, which incidence has increased over the last few years, first in Japan and now in the United States (40, 48). For the same reasons, H$_2$S is also regarded as a possible threat by the Department of Homeland Security (14).

Using a model based on the separation of diffusible H$_2$S determined from alveolar partial pressure and its combined form, a rational frame of reference can be proposed to study how the various antidotes could affect H$_2$S during, and more importantly, following H$_2$S intoxication. Even if 1) the concentrations and kinetics of H$_2$S will remain unknown in humans, and 2) the absolute levels of sulfide may well differ among species, the model developed in this study offers a clinically relevant approach to study the possible benefit of antidotes, in keeping with the acute clinical/respiratory manifestations both during and following H$_2$S exposure.

Conclusions

We have established that 1) H$_2$S is predominantly present in the blood in nondissolved forms, and 2) relatively low concentrations of gaseous H$_2$S were found in the blood when signs of toxicity occurred: around 1 $\mu$M when breathing was stimulated and between 5 and 8 $\mu$M when breathing stopped. These data challenge the use of high micromolar concentrations of exogenous sulfide to study the effects of endogenous sulfide. The combined measurement of C$_4$H$_8$S and CMBBH$_2$S represents an interesting tool and frame of reference that could be used to study the potential effects and benefits of H$_2$S antidotes during and following sulfide exposure.

APPENDIX

H$_2$S Determination in the Blood

Reagents. Monobromobimane (MBB), 1,2-ethanediethyl, HEPES, solid-phase extraction (SFE) columns (silica, 1 g/6 ml), and silica gel (200–400 mesh, 60 Å) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium sulfide was obtained from Alfa Aesar (Ward Hill, MA). Ethyl acetate and hydrochloric acid (0.1 N) were obtained from Fisher Scientific (Pittsburg, PA).

Preparation of standards. Sulfide-dibimane was prepared according to a previously published method (54). Briefly, MBB (0.06 mmol) was dissolved in 250 ml of acetonitrile and added to 750 $\mu$l HEPES (100 mM, pH 8.0) followed by the addition of sodium sulfide (0.03 mmol in water). The reaction mixture was stirred under N$_2$ at room temperature for 50 min followed by extraction with ethyl acetate. The organic extracts were evaporated under vacuum, and the resulting residue was dissolved in chloroform and purified by silica gel column chromatography. The column was eluted progressively with chloroform, 1% methanol, 2% methanol, and 5% methanol in chloroform. The final product eluted in 5% methanol/chloroform. The organic solvent containing the sulfide-dibimane was evaporated under vacuum. The absolute identity of the product was confirmed by proton NMR and mass spectrometry.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.M.K., N.T., B.P., and P.H. performed experiments; C.M.K., N.T., B.P., and P.H. analyzed data; C.M.K. and P.H. interpreted results of experiments; C.M.K., N.T., B.P., and P.H. prepared figures; C.M.K. and P.H. confirmed by proton NMR and mass spectrometry.

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