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

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Klingerman CM, Trushin N, Prokopczyk B, Haouzi P. H₂S concentrations in the arterial blood during H₂S administration in relation to its toxicity and effects on breathing. Am J Physiol Regul Integr Comp Physiol 305: R630–R638, 2013. First published July 31, 2013; doi:10.1152/ajpregu.00218.2013.—Our aim was to establish in spontaneously breathing urethane-anesthetized rats, the relationship between the concentrations of H₂S transported in the blood and the corresponding clinical manifestations, i.e., breathing stimulation and inhibition, during and following infusion of NaHS at increasing rates. The gaseous concentration of H₂S (CgH₂S, one-third of the total soluble form) was computed from the continuous determination of NaHS concentrations in the arterial blood: CMBBH₂S was about 50 times higher than CgH₂S in the arterial blood, and, thus, its concentration, from the measurement of endogenous H₂S and for testing antidotes against its deadly effects.

control of respiration; hydrogen sulfide; toxicity

DURING HYDROGEN SULFIDE (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 and toxicological effects (8). 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 sulfhydryl 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 toxicological standpoint, the combined forms play a dual role since they represent a mechanism of protection (41, 43, 47), trapping H₂S in a non-soluble state, 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 Wittner 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 H$_2$S concentrations using the MBB technique, as well as alveolar H$_2$S partial pressure, we present a quantitative description of the fate of the dissolved and combined forms of H$_2$S 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 H$_2$S toxicity and the debate over the levels of endogenous H$_2$S 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 O$_2$ 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 CO$_2$ and H$_2$S fractions were measured continuously from the second mixing chamber, using a CO$_2$ infrared (Vacumed 17630; Vacumed, Ventura, CA) and H$_2$S (Interscan RM series; Interscan, Simi Valley, CA) analyzer, respectively. The range of the H$_2$S analyzer is 0.001 to 1.00 ppm; at the level of infused H$_2$S used in our study, the expired H$_2$S fraction would be well above this range. In addition, to be able to use this analyzer in a large range of concentrations, an external source of air was introduced into the first mixing chamber in which flow (Vadd) was continuously measured via a second pneumotachograph (1100 series, Hans Rudolph, Shawnee, KS). Vadd was used to determine the actual fraction of H$_2$S in the expired gas (see Measurements and data analysis).

Rats were placed on a heating pad, and body temperature was monitored with a rectal temperature probe (Thermaalert TH-5; Physi-temp, Clifton, NJ).

Catheters (PE-50 tubing) were introduced into the left external jugular or the left femoral vein and in the right femoral artery (the carotid artery was not used to prevent any unnecessary change in the CB or medullary blood flow). The arterial line allowed us to monitor arterial blood pressure (ABP) using a pressure transducer (TA-100; CWE, Ardmore, PA) and to sample blood for H$_2$S measurement and blood gas analysis (see below). The vein was used for NaHS infusion. H$_2$S infusion was stopped just when apex occurred, and animals did resume their breathing activity either spontaneously or, more often, would require mechanical ventilation for a few minutes only.

**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% CO$_2$ for the CO$_2$ analyzer and 0 (air flowing through a charcoal filter) or 0.65 ppm of H$_2$S.

Inspiratory flow, the additional flow of gas delivered (Vadd) to the first mixing chamber. ABE, CO$_2$, and H$_2$S 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 H$_2$S Fraction, Partial Pressure, and Dissolved Concentrations of H$_2$S in the Articular Blood**

The fraction of H$_2$S was continuously measured from the second mixing chamber, defined as FchH$_2$S. Assuming Ve = Vt, the mixed expired H$_2$S fraction (FEH$_2$S) was computed as FEH$_2$S = FchH$_2$S × (Ve + Vadd/Vt). The partial pressure of expired H$_2$S (PEH$_2$S) was then calculated as FEH$_2$S × (PB mmHg).

As the diffusion of H$_2$S is, by definition, only taking place in alveolar regions and not in the pulmonary dead space, the alveolar partial pressure of H$_2$S (PACH$_2$S) was computed as PACH$_2$S = PEH$_2$S × Ve/Va, where Va is the alveolar ventilation. PACH$_2$S was determined from the PECO$_2$/PACO$_2$ ratio (PECO$_2$ × Ve = PACO$_2$ × Vt). PACO$_2$ was estimated from arterial PCO$_2$ (PACO$_2$) sampled at various times during the experiment, while PECO$_2$ was continuously measured from the second mixing chamber.

Pah$_2$S was equated to PACH$_2$S (neglecting the possibility of pulmonary or extra-pulmonary shunts). The concentration of gaseous H$_2$S in the blood (Cgh$_2$S) was calculated as Cgh$_2$S = 0.00012 × Pah$_2$S, with 0.00012 being the coefficient of solubility of H$_2$S (0.09 mol L$^{-1}$/760 mmHg$^{-1}$ at 37°C in saline) (17) (see DISCUSSION). Assuming that H$_2$S is under the form of H$_2$S gas and its sulfhydryl amion HS$^-$ at a ratio of 1/3 and 2/3 in the arterial blood, the concentration of dissolved H$_2$S, CdH$_2$S could be estimated as three times Cgh$_2$S. Finally, the rate of elimination of H$_2$S by the lungs, Vh$_2$S, was determined as FchH$_2$S × (Vadd+Ve).

**H$_2$S Determination in the Blood**

Following a procedure validated by Wintner et al. (54), arterial blood (200 μl) was added with a syringe to a solution of MBB (20 mM in 200 μl of acetonitrile) and 200 μl HEPEs (50 mM, pH 8.0) in a sealed vial. The resulting suspension was stirred for 10 min at room temperature, at which time 100 μl 0.1 N HCl was added to prevent any further reaction between MBB and H$_2$S. The mixture was then extracted (3 × 1 ml) with ethyl acetate, dried over Na$_2$SO$_4$, filtered through glass wool, and the organic solvent was evaporated under vacuum. All of the extraction procedures were completed within 2 h after HCl was added. The residue was dissolved in 1 ml chloroform and purified by SFE. The columns were equilibrated with 20 ml chloroform, and the samples were applied and eluted with 30 ml chloroform, followed by 15 ml of 1% methanol/chloroform and 30 ml of 2% methanol/chloroform. Sulfide-dibimane was eluted in the 2% methanol/chloroform fraction, after being dried under vacuum and redisolved in acetonitrile prior to HPLC analysis.

Samples were analyzed using a Shimadzu HPLC system consisting of two 10AD VP pumps, a SCL-10AVP controller, and a Rhodyne injector, interfaced with a Hitachi L 7485 fluorescence detector. Data were recorded using a Hitachi D2500 integrator. A Phenomenex (Torrance, CA) C-18 Bondclone (4.6 × 300 mm, 10 μm) column was used. Solvent A was 2 mM ammonium acetate, pH 4.0, while solvent B was methanol. The flow rate was 1 ml/min. The following elution program was used: initial conditions 80% A and 20% B, followed by the addition of 2% B for 1 min to 50% B, held for 6 min, and then washed to 100% B for 2 min. The fluorescence excitation wavelength was 390 nm, and the emission wavelength was 470 nm. Under these chromatographic conditions, sulfide-bimane eluted at 19.4 min. The levels of sulfide-bimane in rat blood were determined on the basis of

**REFERENCE**

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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 Tcep(2-carboxymethyl)phosphine hydrochloride (Tcep; Soltec Bio Science, Beverly, MA). The rationale was that in the presence of the reducing agent Tcep, which should limit the sulfhydration 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 to remain higher than in the blood if CMBBH2S is primarily influenced by the combination of H2S with hemoglobin. Samples were incubated in hermetically sealed, glass vials and processed by the MBb method, as described in the previous paragraphs. The samples were analyzed for CMBBH2S after 10 min of contact between H2S and the various solutions.

**Protocol**

**NaHS infusion.** NaHS (sodium hydrosulfide hydrate; Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration corresponding to 10.5 μmol/ml (10.5 mM). H2S was prepared minutes before the infusion and kept in airtight syringes. Infusion rates were determined on the basis of a series of pilot experiments not included in the present data. The solution was infused intravenously starting at a rate of 2.09 μmol/min using an infusion pump (Fusion 100; Chemyx, Stafford, TX). The flow rate was increased by about 2 μmol/min every 4 min until an apnea occurred. H2S administration was stopped as soon as breathing ceased. Apneic periods were defined as breaths with an increase in TTOT by more than 5 times (see RESULTS). While FAH2S was measured continuously, blood was sampled at the third min into each infusion rate. After the animal recovered from the apneic period and when minute ventilation was back to baseline, a new infusion of a sublethal level of H2S (4.42 μmol/min) was used for 5 min, and blood was sampled again during the last minute of the infusion period, and then at 5, 10, and 15 min into recovery.

**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 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.

**Statistics**

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 CMMBH2S at the different rates of infusion were compared using one-way ANOVA. For the in vitro experiments, data 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 H2S in the Expired Gas**

Effect of the time of contact between H2S and the expiratory gas and effect of gas composition. We found no difference between the concentrations of the H2S in air, N2, or 100% O2 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 H2S 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 Vadd on the determination of FH2S in the mixing chamber where the expired gas was sampled by our H2S analyzer, the effects of the flow of a gas with a known concentration of H2S in N2 was evaluated in keeping with the levels of Vadd that we used in vivo (Fig. 2). FH2S 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.

**CgH2S and CMBBH2S During H2S Infusion: Effects on Breathing**

Figure 3 is an example of the change in the alveolar fraction of H2S at various rates of NaHS infusion along with ventilation. H2S was undetectable in its soluble form in baseline conditions, while CMBBH2S averaged 0.7 ± 0.5 μM. Clearly, CgH2S increased according to the level of H2S infused, and CMBBH2S followed a very similar pattern, but at a level that was about 50 times that of CgH2S at low concentrations and 5 or 6 times that of the lethal level. The average values of CgH2S and CMBBH2S, as a function of the rate of H2S infusion, are displayed in Figs. 4 and 5.

CgH2S was found to average 1.1 ± 0.7 μM when breathing was stimulated (H2S partial pressure of 9.5 10−3 ± 5.5 10−3 mmHg), corresponding to a CMBBH2S 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 mol/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 CgH2S and 25.4 and 50.9 μM for CMBBH2S, 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 H2S by about 8 times, while CMBBH2S 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, CH2S 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 H2S 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, CgH2S dropped dramatically. The kinetics of this response was indiscernible from the off-transient of the analyzer. CMBBH2S

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Fig. 2. A: fraction of H2S was recomputed from the data obtained with our H2S analyzer using a calibration gas containing H2S in nitrogen (25 ppm) diluted in air to remain within the range of the analyzer (see METHODS). H2S was delivered and mixed with the flow of air in the first chamber, while tubing was interposed between the first and second chamber, allowing a time of contact between H2S and the air of 600 ms (short circuit, SC) or 1.5 s (long circuit, LC). The time of contact had no effect on the concentration of gaseous H2S. B: changing the composition of gas introduced into the respiratory circuit, (N2, 100% O2, or air), which mixed with the H2S calibration gas, had no effect on H2S concentration. C: effects of increasing the flow of a gas with an original FH2S of 0.65 ppm on the measured H2S fraction. D: relationship between FH2S and additional air flow, Vadd, in absolute levels (closed circles) and % of change. Note that there was a small, but systematic, increase in H2S determination by 6% when flow was increased from 0 up to 25 l/min.
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 μM for CgH₂S and 11.1 μM 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.

Fig. 3. Recording from a rat receiving an incremental rate of H₂S infusion from 2.09 to 10.46 μmol/min. Inspiratory flow (V), minute ventilation (V̇I), additional airflow (Vadd), and the alveolar fraction of H₂S (FAH₂S) are displayed. The symbol * indicates when arterial blood was sampled for CMBBH₂S determination. Minute ventilation was stimulated (first arrow) only when the rate of H₂S infusion was 6.10 μmol/min. Note that this increase in breathing was associated with a production of augmented breaths (sighs) along with periodic breathing. FAH₂S increased along with the rate of NaHS infused. At the highest rate, breathing decreased dramatically (second arrow), and then an apnea occurred. In this example, infusion was stopped, allowing breathing to slowly recover following 30 s of mechanical ventilation (not shown).

Fig. 4. Concentration (mean ± SD) of the gaseous form of H₂S in the arterial blood (CgH₂S; open bars) and of H₂S measured in the blood using MBB (CMBBH₂S; solid bars). Increasing the rate of NaHS infusion caused an increase in CgH₂S and CMBBH₂S. The majority of H₂S found in the blood was not in the dissolved form. CgH₂S increased from zero to a maximum of 8.8 μM. Baseline CMBBH₂S averaged 0.74 μM and increased up to 50.88 μM, which represents the lethal level of H₂S. **CgH₂S significantly different from baseline at P < 0.001. **Significantly different from CgH₂S at P < 0.001.
μM, respectively. These results complement the data previously published on the concentrations of H$_2$S in the blood using the MBB technique in the rat (54) and also offer a simple way to estimate the concentrations of dissolved H$_2$S in the blood based on the continuous determination of alveolar H$_2$S 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 H$_2$S, as well as the potential impacts of antidotes against H$_2$S 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 H$_2$S and MBB to determine the change in H$_2$S concentrations in the blood during H$_2$S 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 H$_2$S 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 CMBBH$_2$S increased sharply, with a very steep slope, when the rate of H$_2$S administration was close to the lethal levels of exposure, corresponding to a CMBBH$_2$S around 30–40 μM. Wintner et al. (54) also showed, using a series of in vitro studies, that the MBB technique can identify H$_2$S present in various forms in the blood. First, H$_2$S concentrations in the plasma were similar to the values obtained using amperometry, which only measures soluble H$_2$S (15, 30, 37). We found that in saline, CMBBH$_2$S did not change within 10 min, if evaporation of H$_2$S was prevented, in keeping with our previous results (49, 50). In addition, since H$_2$S is present in the form of HS$^-$ (70–80%) and H$_2$S (30–20%) at physiological pH in PBS, CMBBH$_2$S includes all forms of dissolved H$_2$S (gaseous and sulfhydryl anion HS$^-$).

Perhaps more importantly, these authors reported that when exogenous H$_2$S was added to whole blood, it disappeared virtually within seconds in its soluble form, as determined by amperometry, while CMBBH$_2$S remained elevated for more than 20 min, subsiding progressively with time. The dissociation between the concentrations obtained from amperometry and from the MBB method in the whole blood, was not observed in the plasma (54). Although the exact nature of the H$_2$S sink measured by MBB was not described in these studies, we found that CMBBH$_2$S was higher when Hb was replaced by Methb. The latter has a much higher affinity for H$_2$S than its ferrous counterpart. Finally, adding the reducing agent TCEP (1 mM concentration) prevented the decrease in H$_2$S concentrations in the plasma but not in the blood. Taken together, the data from Wintner et al. (54) using amperometry vs. the MBB technique in the blood and plasma combined with our present results, suggest that 1) the MBB method does not only allow...
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 interact-
ing 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 elec-
trode. As pointed out in the previous paragraph, the concen-
trations 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 Ve/V_A),
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 tradi-
tional 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 vari-
able dilution related to this nonstable flow and, more import-
antly, 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 assum-
ing 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 Setchenow coefficient of 0.064 (13) [H = H0e$^{-0.064\cdot M}$].
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 concentra-
tions 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 tech-
nique 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 picomolar 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 deter-
dined: 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 elev-
ated 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
apneic 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

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**References**

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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 µM for breathing stimulation and less than 8 µM for the cessation of breathing, i.e., 3 and 24 µM 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 µM (39) or the 300 µM 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 to public health. 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).

APPENDIX

H$_2$S Determination in the Blood

Reagents. Monobromobimane (MBB), 1,2-ethanedithiol, 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 µl 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 identity of the product was confirmed by proton NMR and mass spectrometry. NMR (CDCl$_3$)δ 3.8 (s, 4H, CH$_2$), 2.3 (s, 6H, CH$_3$), 1.9 (s, 6H, CH$_3$), 1.85 (s,6H, CH$_3$); m/z (M + H) 415.

1,2-Ethanediethyl-dibimane was synthesized as follows: 1,2-ethanediethyl (0.6 mmol) was dissolved in 500 µl of acetonitrile. 500 µl of HEPES (5 mM, pH 8.0) was added to this solution followed by 0.25 mmol MBB in 1.25 ml of acetonitrile. The solution was stirred under N$_2$ at room temperature for 2 h and extracted with ethyl acetate. The organic extracts were dried over Na$_2$SO$_4$, and the organic solvent was evaporated under vacuum. The resulting product was purified by silica gel column chromatography in a similar manner as described above for purification of sulfide-dibimane. The 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. interpreted data; C.M.K., N.T., B.P., and P.H. approved the final version of the manuscript; P.H. conceived and designed the research.

REFERENCES

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47. Truscott A. Suicide fad threatens neighbours, rescuers. CMAJ 179: 312–313, 2008.


