

In Vivo Interactions Between Cobalt or Ferric Compounds and the Pools of Sulphide in the Blood During and After H₂S Poisoning

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ABSTRACT

Hydrogen sulphide (H₂S), a chemical hazard in oil and gas production, has recently become a dreadful method of suicide, posing specific risks and challenges for the first responders. Currently, there is no proven effective treatment against H₂S poisoning and its severe neurological, respiratory or cardiac after-effects. We have recently described that H₂S is present in various compartments, or pools, in the body during sulphide exposure, which have different levels of toxicity. The general goals of our study were to (1) determine the concentrations and kinetics of the various pools of hydrogen sulphide in the blood, i.e., gaseous (CgH₂S) versus total sulphide, i.e., reacting with monobromobimane (CMBBH₂S), during and following H₂S exposure in a small and large mammal and (2) establish the interaction between the pools of H₂S and a methemoglobin (MetHb) solution or a high dose of hydroxocobalamin (HyCo). We found that CgH₂S during and following H₂S infusion was similar in sedated sheep and rats at any given rate of infusion/kg and provoked symptoms, i.e., hyperpnea and apnea, at the same CgH₂S. After H₂S administration was stopped, CgH₂S disappeared within 1 min. CMBBH₂S also dropped to 2–3 μM, but remained above baseline levels for at least 30 min. Infusion of a MetHb solution *during* H₂S infusion produced an immediate reduction in the free/soluble pool of H₂S only, whereas CMBBH₂S increased by severalfold. HyCo (70 mg/kg) also decreased the concentrations of free/soluble H₂S to almost zero; CgH₂S returned to pre-HyCo levels within a maximum of 20 min, if H₂S infusion is maintained. These results are discussed in the context of a relevant scenario, wherein antidotes can only be administered *after* H₂S exposure.

Hydrogen sulphide (H₂S) intoxication is one of the most common causes of death following gas exposure in the workplace (Beauchamp *et al.*, 1984; Fuller and Suruda, 2000; Guidotti, 1996; Reiffenstein *et al.*, 1992). Although these intoxications remain rare, H₂S is extremely toxic at relatively low concentrations (Fuller and Suruda, 2000) leading to a fatal apnea and cardiac arrest within a few minutes at only 0.1% (Beauchamp *et al.*, 1984; Guidotti, 1996; Reiffenstein *et al.*, 1992), corresponding to concentrations in the blood of only 5–8 μM of gaseous sulphide (Klingerman *et al.*, 2013). H₂S remains one of the primary chemi-

cal hazards in oil and gas production, well drilling and gas refining industries (Arnold *et al.*, 1985; Costigan, 2003; EPA, 2003). H₂S has also been more recently used as a method of suicide first in Japan and now in the United States, by mixing in a closed environment—typically in a car—a source of sulfur and various types of acidic solutions readily available in most discount stores. This method of suicide has increased over the past few years, creating new risks not only for those intent on harming themselves, but also for first responders and neighbors (Reedy *et al.*, 2011; Truscott, 2008).

The toxicity of H₂S/HS⁻ is to be accounted for by its various effects on cellular function, the most important being its impediment of ATP formation via the inhibition of cytochrome c oxidase (Cooper and Brown, 2008; Dorman et al., 2002). Neurons, unable to oxidize H₂S (Lagoutte et al., 2010), are particularly vulnerable (Almeida and Guidotti, 1999; Almeida et al., 2008; Greer et al., 1995; Tvedt et al., 1991).

Various agents have been proposed to treat H₂S poisoning, some with the theoretical purpose of “oxidizing” H₂S (e.g., hyperoxia) (Bitterman et al., 1986; Haouzi et al., 2011a; Smilkstein et al., 1985; Smith et al., 1976), trapping it with metallo-compounds (e.g., ferric iron produced by nitrite-induced methemoglobinemia or cobalt in vitamin B12) (Haouzi et al., 2011b; Smith, 1969; Smith et al., 1976; Truong et al., 2007; Van de Louw and Haouzi, 2012b) or removing it from cysteine residues using reducing agents (Warenycia et al., 1990). Finally, sodium bicarbonate and glucose have been shown to exert protective effects, based on an interesting series of observations (Almeida and Guidotti, 1999; Guidotti, 2010). There is still no consensus on the strategy to be used. More importantly, as described in the following paragraph, H₂S is present in various forms in the blood and tissues, i.e., diffusible versus combined, which have very different toxic effects. Little is known about the interactions of these antidotes with the various pools of H₂S responsible for sulphide toxicity. Perhaps more importantly, the efficacy of any antidotes used against H₂S poisoning is eventually dictated by the fate of these various pools of H₂S in the blood and tissues “after” H₂S exposure, i.e., when patients are withdrawn from the source of intoxication and taken care of by paramedics or later on in the emergency room.

We have previously used a simple model to describe the various compartments of H₂S in the blood of the rat during exogenous H₂S administration (Klingerman et al., 2013). Briefly, in the rat, H₂S infusion at a rate 2 μmol/min produces no visible effects on blood pressure or breathing, whereas at 10 μmol/min a terminal apnea occurs in minutes. Within this range, exogenous H₂S is present in the blood in two different forms, displaying an exponential relationship with the dose administered (Klingerman et al., 2013). These two distinct pools of sulphide can be described as (1) a pool of “free” or diffusible H₂S that includes (a) H₂S in gaseous form (CgH₂S), whose partial pressure in the arterial blood is in equilibrium with the alveolar gas and with all tissues (Carroll and Mather, 1989), and (b) the sulfhydryl anion, HS⁻ (Almgren et al., 1976; Millero, 1986), and (2) a pool of combined H₂S primarily composed of sulphide fixed on the ferrous iron of hemoglobin (Hb) and proteins in the plasma (Haggard, 1921; Smith, 1967; Smith and Gosselin, 1966; Wintner et al., 2010). The pool of gaseous H₂S in the arterial blood can be estimated based on H₂S alveolar partial pressure (Klingerman et al., 2013) or via direct measurement by amperometry (Wintner et al., 2010), whereas the total amount of H₂S in the blood could be approached by a more global method such as the derivation of monobromobimane (MBB) (Wintner et al., 2010). H₂S, being extremely soluble, in its gaseous form (Barrett et al., 1988; Carroll and Mather, 1989; De Bruyn et al., 1995; Douabul and Riley, 1979) diffuses with virtually no delay between the blood and the tissues and within the cells. HS⁻ and the pool of combined sulphide are present at much higher concentrations than the gaseous form in the blood. Within the cells, H₂S/HS⁻ combines with various metallo-proteins (e.g., the ferrous iron of Hb) or proteins (e.g., cysteine residues) (Mustafa et al., 2009; Warenycia et al., 1990). The majority of H₂S oxidation takes place in the mitochondria (Bouillaud and Blachier, 2011; Lagoutte et al., 2010), where H₂S also expresses its main toxicity by blocking the cy-

tochrome c activity and thus ATP formation. The rate of elimination of H₂S *in vivo* is not established as far as these various pools of H₂S are concerned. Yet this question is of major importance as it represents a prerequisite for offering a strategy of treatment for H₂S poisoning. This question also resonates with the increasing number of studies, which have been using IV or IP injections of H₂S in various animal models to reduce the inflammatory cascade and the oxidative stress produced by a hypoxic, ischemic or infectious insult (see Olson, 2011b, Szabo, 2007, and Whiteman and Winyard, 2011 for review and discussion).

The present study intends to address the following questions: (1) what is the rate of disappearance of the various forms (free, combined) of sulphides, after exogenous H₂S administration in the rat, in keeping with the level of H₂S infused and is there evidence for persistence of H₂S (and under which form) after the cessation of exposure/injection? (2) To which extent can the data obtained in the rat on the various pools of H₂S and their kinetics be transposed to a larger mammal more closely resembling the size of a human being? (3) How two potential H₂S antidotes, i.e., a methemoglobin (MetHb) solution (600–1200 mg/kg) or a high dose (70 mg/kg) of hydroxocobalamin (HyCo), affect the pools of H₂S, and could these interactions be reconciled with their use during and, more importantly, “following” H₂S exposure?

Our main objective was to establish a rational frame of reference, which can be used to understand the short- and long-term effect, or the lack thereof, of an acute exposure to H₂S and to define if a window of opportunity exists during which MetHb and/or HyCo could be effective as H₂S antidotes in keeping with sulphide kinetics.

MATERIALS AND METHODS

1. Rat Experiments

Materials and Methods. The experimental procedures were performed on adult, male, Sprague Dawley rats (616 ± 0.14 g, 45 ± 2 weeks old). All procedures received prior approval from the Pennsylvania State University College of Medicine Institutional Care and Use Committee. Anesthesia was induced with 3.5% isoflurane in O₂ followed by an intra-peritoneal injection of 1.2 g/kg urethane as previously described (Haouzi and Van de Louw, 2013). Rats were placed on a heating pad and body temperature (BT) was monitored with a rectal temperature probe (Thermalert TH-5, Physitemp, NJ) and kept between 36–38°C. The rats were tracheostomized and a catheter (14 g, 2.25 mm OD) was placed in the trachea. Catheters (PE-50 tubing) were introduced into the left external jugular vein and the right femoral artery. The jugular vein was used for NaHS infusion. At the end of the experiment, rats were euthanized with CO₂ followed by aortic dissection.

Measurements. The tracheal catheter was connected to a Hans Rudolph low dead space two-way valve as previously described (Klingerman et al., 2013). The inspiratory port of the valve was connected to a pneumotachograph (1100 Series, Hans Rudolph, KS) to measure inspiratory flow. The expiratory valve was connected to two 5 ml “mixing chambers” placed in series. The outlet of the second chamber was connected to a filter containing activated charcoal. Mixed expired CO₂ and H₂S fractions were measured continuously from the second mixing chamber using CO₂ infrared (Vacumed model 17630, Ventura, CA; 0–10%) and H₂S (Interscan RM series, Simi Valley, CA; 0.001–1 ppm) analyzers. The gas analyzers were calibrated immediately before use

with different gas mixtures containing a known concentration of CO₂ in air or H₂S in N₂.

An external source of air was introduced into the first mixing chamber and gas flow (\dot{V}_{add}) was continuously measured using a pneumotachograph (1100 Series). This approach has been described in detail elsewhere (Klingerman et al., 2013).

Inspiratory flow, \dot{V}_{add} , arterial blood pressure (ABP), CO₂ and H₂S fractions, along with rectal temperature signals, were digitized at 200 Hz using an analog to digital data acquisition system (Powerlab 16/35, AD Instruments, Colorado Springs, CO). 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 (\dot{V}_I) was computed as $f \times VT$. The arterial line was connected to a pressure transducer (TA-100, CWE Inc., PA) to monitor ABP and was used for blood sampling.

2. Sheep Experiments

Materials and Methods. Spontaneously breathing, sedated, adult Dorset sheep (*Ovis aries*, ewes, 1–2 years old) weighing 54 ± 13 kg were used in these experiments. The animals were pre-medicated with ketamine (20 mg/kg) IM and then anesthesia was induced with an IV loading dose of sodium thiopental (Pentothal 10 mg/kg). Anesthesia was subsequently maintained by an IV solution containing urethane (80 mg/kg) and alphachloralose (15 mg/kg), as previously described (Haouzi et al., 2011a). The infusion of urethane-chloralose was repeated every 2 h throughout the experiment.

The animals were tracheostomized and an inflatable-cuff tracheal cannula (Shiley No. 7) was inserted through the tracheostomy. Systemic ABP and heart rate were monitored by placing a catheter in one of the carotid arteries, which was also used for blood samples. Venous catheters (Plastimed 3F) were placed in one jugular and one femoral vein for injection of anesthetic agent or antidotes.

Measurements. Respiratory flow and the pulmonary gas exchange rate were recorded with a Hans-Rudolph pneumotachograph (Hans-Rudolph, Kansas City, MO) connected to the tracheostomy tube and to a differential pressure transducer. The expiratory valve was connected to a mixing chamber where the mixed expired O₂ and CO₂ fractions were measured continuously using O₂ (Oxystar-100, CWE) and CO₂ (model 17630, VacuMed, Ventura, CA) analyzers. H₂S expired fractions were also measured continuously from the same mixing chamber (Interscan RM series, Simi Valley, CA; 1–200 ppm). The gas analyzers were calibrated immediately before use with different gas mixtures containing a known concentration of CO₂ and O₂ in air and H₂S in N₂. ABP and respiratory flow signals were digitized at 200 Hz (Power Lab system). The respiratory flow signal was integrated for breath-by-breath calculation of tidal volume and minute ventilation, oxygen consumption and CO₂ production. At the end of the experiment, animals were euthanized by lethal injection of barbiturate (Pentothal 200 mg/kg) in the jugular catheter.

3. Determination of Dissolved Concentrations of H₂S in the Arterial Blood

In the rat, expired H₂S was determined as previously described (Klingerman et al., 2013). Briefly, the fraction of H₂S was continuously measured from the second mixing chamber (FchH₂S). Then, the mixed, expired fraction of H₂S (F_EH₂S) was computed as $FchH_2S \times (\dot{V}_I + \dot{V}_{add}/\dot{V}_I)$. The partial pressure of expired H₂S (P_EH₂S) was then calculated as $F_E H_2S \times (PB - PH_2O)$, allowing the

determination of the alveolar partial pressure of H₂S (P_AH₂S) as $P_A H_2S = P_E H_2S \times \dot{V}_E/\dot{V}_A$, whereas \dot{V}_E/\dot{V}_A was determined from the P_ECO₂/P_ACO₂ ratio. \dot{V}_E was considered equal to \dot{V}_I , whereas P_ACO₂ was estimated from arterial PCO₂ (PaCO₂) sampled at various times during the experiment, whereas P_ECO₂ was continuously measured from the second mixing chamber. PaH₂S was assimilated to P_AH₂S and the concentration of gaseous H₂S in the blood was calculated as $CgH_2S = 0.00012 PaH_2S$, with 0.00012 being the coefficient of solubility of H₂S (0.09 mole/L/760 mm Hg⁻¹ at 37°C) (see Klingerman et al., 2013 for details and references). Following the assumption that H₂S is under the form of H₂S gas and HS⁻ at a ratio of 30 and 70%, respectively, at a pH of 7 (Almgren et al., 1976.; Millero, 1986), the total concentration of dissolved H₂S (CdH₂S) was computed as $3 \times CgH_2S$.

In the sheep, the same measurements were performed, but as no additional flow was used, F_AH₂S was simply determined as $P_A H_2S = P_E H_2S \times (\dot{V}_E/\dot{V}_A)$.

4. Measurement of H₂S in the Blood Using Monobromobimane

Monobromobimane (MBB), 1,2-ethanedithiol, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (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 sulphide was obtained from Alfa Aesar (Ward Hill, MA). Ethyl acetate and hydrochloric acid (0.1 N) were purchased from Fisher Scientific (Pittsburg, PA).

An extensive description of the preparation of the internal standard and various validations of this method has been presented in details elsewhere (Klingerman et al., 2013), following the method used by Wintner et al. (Wintner et al., 2010). Briefly, venous blood (200 μl) was added to a solution of MBB (20mM in 200 μl of acetonitrile) and 200 μl HEPES (50mM, pH 8.0) in a sealed vial. Samples were analyzed using a HPLC system (Shimadzu) with a Phenomenex (Torrance, CA) C-18 Bondclone (4.6 × 300 mm, 10 μm) column. Under these chromatographic conditions, sulphide-bimane eluted at 19.4 min. The levels of sulphide-bimane in rat blood were determined based on standard curves constructed for these analyses.

As a control, 200 μl of a MetHb solution (600 mg/ml saline) was reacted with MBB as described for the blood samples. HPLC analysis of this sample showed no interference with the detection of the sulphide-bimane. The components of H₂S measured by these methods includes both the dissolved form of H₂S and the part combined to the red cell components of the blood and plasma proteins (Klingerman et al., 2013; Olson, 2011a; Wintner et al., 2010).

5. Methemoglobin Concentration in the Blood

Arterial gas partial pressure in O₂ and CO₂ and MetHb concentrations were determined using a GEM Premier 4000 gas analyzer (Instrumentation Laboratory, Bedford, MA). The accuracy of the measurement of MetHb was previously validated using a spectrophotometric method in duplicate (Rahman et al., 2009). This technique was also used to determine the concentration of MetHb in the Hb solution, which was found to be about 98% (Van de Louw and Haouzi, 2012).

6. Protocol

Rat Experiments. NaHS infusion and recovery (12 rats): First, NaHS (sodium hydrosulphide hydrate, Sigma Aldrich, St Louis, MO) was dissolved in sterile saline at a concentration of 0.8 mg/ml, without exposure to air, assuming an equimolar conversion of NaHS into H₂S/HS⁻ (see last paragraph of method section for further details) after taking into account 25% hydration of

NaHS. H₂S was infused into the jugular vein using an infusion pump (Chemyx Inc., Model Fusion 100, Stafford, TX) at a rate ranging from 2.8 to 10.2 μmol/min corresponding to concentrations that ranged from levels below the threshold for ventilatory stimulation to levels producing apnea. These doses were chosen based on our previously published data (Klingerman et al., 2013). For the rates above 8 μmol/min, which produce an apnea after 2–3 min, all animals were ventilated and the circuit was modified to be able to collect the expiratory gases. CMBBH₂S was measured before H₂S infusion, at the end of infusion, then at 1, 3, 5, 10, 15 and 30 min following the cessation of exposure.

Methemoglobin infusion (10 rats): A MetHb solution of 600 mg of human-derived Hb (Sigma Aldrich) in sterile saline (200 mg/ml) was infused slowly over about 1 min (30–90 s) into the femoral artery. It was determined in pilot experiments that the inhibitory effect of MetHb infusion on H₂S-induced breathing simulation was similar whether it was administered into the femoral vein or artery. For this experiment, we chose to infuse into the artery to prevent a direct mixture with NaHS in the venous system. This dose was calculated based on an average endogenous Hb concentration (130 g/l) and blood volume (80 ml/kg) to reach a level of MetHb around 8%. This had been previously confirmed by pilot experiments in two rats, which were not included in the present data analysis. This protocol was performed in 10 rats. In three of these rats, CgH₂S, CdH₂S, and CMBBH₂S were also measured 5, 10, and 15 min following the cessation of NaHS infusion.

Effect of withdrawal and infusion of whole blood versus methemoglobin solution on H₂S concentration (3 rats): Because infusion of several milliliters of MetHb over 30–90 s alters the volemia and therefore could affect the rate of interaction between H₂S and Hb, we sought to delineate the effects of changing blood volume (or Hb) per se. CgH₂S was determined in four additional rats during (1) withdrawing and then injecting a 2 ml volume of arterial blood and (2) the injection of 2 ml of MetHb solution (150 mg/ml), corresponding to the baseline Hb concentration. We withdrew and then injected arterial blood twice before MetHb infusion and then two more times after.

Sheep Experiments. NaHS infusion and recovery (5 sheep): To limit the number of animals used, H₂S was infused at five different levels in a ramp-like manner, following the same approach used previously in the rat (Klingerman et al., 2013). H₂S was prepared a few minutes before the infusion and kept in airtight syringes. The solution was infused intravenously into the femoral vein using an infusion pump (Fusion 100; Chemyx Inc, Stafford, TX). The flow rate was increased from 135 to 665 μmol/min every 5 min by steps of 135 μmol/min, until an apnea occurred. This protocol was chosen based on our previous data and a series of pilot experiments, to cover a range of H₂S exposure from no obvious symptoms to apnea, similar to the current experiments in the rat. H₂S administration was stopped as soon as breathing ceased, and then the animals were mechanically ventilated (see the Results and Discussion sections for further details). The expiratory circuit allowed us to measure F_EH₂S continuously during and following exposure, whether during spontaneous or mechanical ventilation. During mechanical ventilation, the time constant of the changes in F_EH₂S was longer than during spontaneous breathing by 20 s, which was taken into account for the determination of F_AH₂S. Blood was sampled at the third minute into each infusion rate and then at 5, 10, and 15 min into recovery.

Methemoglobin and hydroxocobalamin infusion in the sheep: All of the sheep recovered from the apneic period, and

minute ventilation was back to baseline within 10–15 min. Then a new infusion of a sub-lethal level of H₂S (270–405 μmol/min) was initiated into the femoral vein for 3 min. Next, a solution of HyCo (Cyanokit 70 mg/kg) was infused slowly over about 2–3 min in a jugular vein and the level of F_AH₂S was monitored up to 25 min while H₂S infusion was maintained.

Fifty to sixty minutes after the end of H₂S infusion, when the ventilatory and circulatory parameters were back to baseline (including expired H₂S, see the Result section), a second infusion of a sub-lethal level of H₂S at the same level was resumed and MetHb solution was injected. Similar to the rat, the dose of MetHb was computed based on an average endogenous Hb concentration (130 g/l) and blood volume (80 ml/kg) to reach a level of MetHb around 8%. Then H₂S was determined again following at the end of H₂S infusion at 5, 10 and 15 min.

Injections were performed in the same animal, to minimize the number of sheep studied, assuming that all H₂S in free forms had disappeared from the blood before a new trial (see the Discussion section for more details).

7. Data Analysis

All data are expressed as mean ± SD. The relationship between alveolar H₂S (CgH₂S) and total H₂S in the blood (CMBBH₂S) was established in keeping with the dose infused and the level of minute ventilation. Regression analyses using a linear, exponential, or power function were used to describe the relationship between CH₂S and the rate of H₂S infusion in both species and the best coefficients of regression are reported.

The concentration of H₂S at which breathing was increased by 30% was chosen as the threshold for carotid body (CB) stimulation (H₂S-induced respiratory symptoms). CgH₂S as well as CMBBH₂S at the different rates of infusion were compared using one-way analysis of variance (ANOVA); *p* < 0.05 was considered as significant. Kinetics of recovery of CgH₂S and CMBBH₂S were described as follows: the time constant of the response was established as the time required for CgH₂S to drop by 64% after subtracting the time constant of the analyzer and the circuit. CgH₂S and CMBBH₂S during recovery were compared using Friedman Repeated Measures Analysis of Variance, followed by a post-hoc, pairwise, multiple comparison procedure (Bonferroni).

A paired t-test was used to compare CgH₂S, CdH₂S, and CMBBH₂S before MetHb infusion and then 3 min after MetHb administration during NaHS infusion. A paired t-test was also used to compare the change in MetHb levels before and 3 min after MetHb infusion. The effects of hydroxocobalamin before, 3 min and 20 min after the end of injection were compared using a one-way ANOVA with repeated measurements. The effects of blood pressure on the H₂S concentrations were compared by Friedman Repeated Measures Analysis of Variance, followed by a post-hoc, pairwise, multiple comparison procedure (Bonferroni). *p* < 0.05 was considered as significant.

8. Symbols and Conventions Used to Describe the Various Pools of Sulphide in the Blood and in Solution

H₂S can refer to various and very different pools of sulphide: (1) its gaseous form, (2) H₂S in solution (dissolved H₂S), which at pH 7 comprises 70–80% HS⁻ and 20–30% gaseous H₂S, and (3) H₂S present in combined forms (metallo-proteins or on cysteine residues) in a biological milieu, including the blood. For consistency and clarity, the symbol CgH₂S will be used to refer to the concentration of the gaseous form of H₂S in solution and CdH₂S will refer to the concentration of dissolved H₂S, comprising H₂S in gaseous form and HS⁻. NaHS, when in a saline solution, dissociates into gaseous H₂S and HS⁻, so that an infusion of NaHS

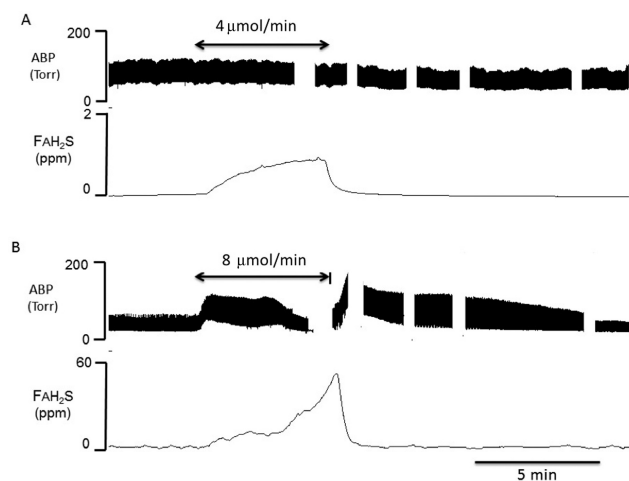


FIG. 1. Example of recordings obtained in two rats showing the effect of intravenous H_2S infusion for 5 min on $\text{F}_A\text{H}_2\text{S}$ (proportional to CgH_2S) at a dose producing no effect on breathing ($4 \mu\text{mol}/\text{min}$, upper panel) and at a level producing an apnea ($8 \mu\text{mol}/\text{min}$, lower panel). In the latter, the rat was mechanically ventilated. $\text{F}_A\text{H}_2\text{S}$, which was determined based on minute ventilation, $\text{F}_E\text{H}_2\text{S}$, and PaCO_2 (see the Materials and Methods section for determination) increased as a function of the levels of H_2S infused. Note the lack of a cardiovascular effect at the lowest infusion rate whereas at the highest level, $\text{F}_A\text{H}_2\text{S}$ continues to rise until blood pressure starts to drop. Interruptions in the BP trace correspond to periods of blood sampling.

will contain an equimolar concentration of $\text{H}_2\text{S}/\text{HS}^-$ (with 70% of HS^- and 30% gaseous H_2S , the latter evaporating rapidly if syringes are not sealed or contain air). No combined forms of H_2S are present in saline. Infusion of sulphide will be expressed as infusion of NaHS (and not $\text{H}_2\text{S}/\text{HS}^-$) in keeping with the way our solutions of sulphide were prepared. Total concentration of sulphide in the blood, determined by the MBB method, will be expressed as CMBBH_2S corresponding to any sulphide reacting with MBB, comprising the soluble and combined forms of H_2S .

RESULTS

NaHS Infusion and Recovery in the Rat

NaHS was infused at only one rate in any given rat ($n = 12$). The rate of infusion was randomly selected between 2.8 and 10.2 $\mu\text{mol}/\text{min}$ and applied for 5 min (Fig. 1). As developed in the Materials and Methods section (Protocol), the lowest levels were below the threshold of breathing stimulation (no clinical signs), whereas the highest rate produced an apnea and a drop in blood pressure. Below 6 $\mu\text{mol}/\text{min}$, CgH_2S remained stable during infusion suggesting that the rate of disappearance (oxidation) was similar to the rate of infusion; at higher levels, no steady state could be attained and CgH_2S continued to rise until the cessation of infusion (Fig. 1). CgH_2S was found to range between 0.4 μM and 15.2 μM in keeping with the level of H_2S infused. These values corresponded to levels of total dissolved H_2S concentrations (CdH_2S) ranging from 1.2 to 50 μM , while CMBBH_2S ranged from 4.9 to 87.1 μM . For clarity, concentrations are also displayed as a function of three levels of infusion in Figure 2, high (H: $8.9 \pm 1.2 \mu\text{mol}/\text{min}$), moderate (M: $7.9 \pm 2 \mu\text{mol}/\text{min}$), and low (L: $5.1 \pm 2.4 \mu\text{mol}/\text{min}$), whereas individual data are shown in Figure 3. The relationship between H_2S concentrations and the rate of H_2S infusion was very similar to the relationship established in another group of rats previously studied during a ramp-like increase in H_2S infusion rate (Klingerman et al., 2013).

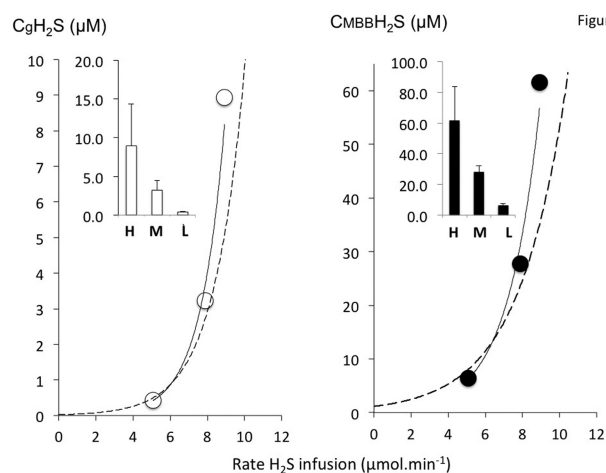


FIG. 2. Concentrations of gaseous H_2S in the rat (CgH_2S , left panel) and total H_2S (CMBBH_2S , right panel) at various infusion rates. For clarity, concentrations were displayed as a function of three levels of infusion including high (H: $8.9 \pm 1.2 \mu\text{mol}/\text{min}$), moderate (M: $7.9 \pm 2 \mu\text{mol}/\text{min}$), and low (L: $5.1 \pm 2.4 \mu\text{mol}/\text{min}$). The relationships between CH_2S and H_2S rate of infusion (shown in dotted lines with the best exponential fits) are very similar to the relationship we previously established in another group of rats during a ramp-like increase in H_2S infusion rate. CMBBH_2S was determined by HPLC/fluorescence analysis of MBB-S-MBB formed from incubation of MBB reagent with the blood of the rat. CgH_2S represents a small proportion of total CH_2S , whereas both concentrations increase sharply as the rate increases. Increasing the rate of infusion in the low range by only 2 $\mu\text{mol}/\text{min}$ (20–30%) results in an increase in the gaseous form of H_2S and CMBBH_2S by more than 2–3 times.

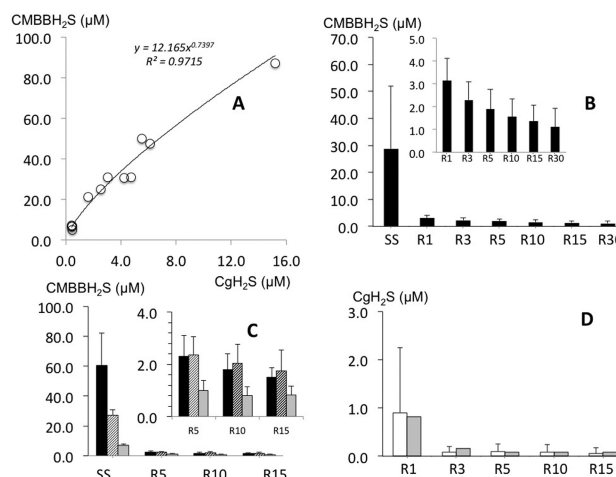


FIG. 3. (A) Relationship between CgH_2S and CMBBH_2S during H_2S infusion obtained in all rats. (B) CMBBH_2S (mean \pm SD) before the cessation of H_2S infusion and at 1, 3, 5, 10, 15 and 30 min into recovery. Note that within 1 min, CMBBH_2S dropped to a level of H_2S that averaged 3.1 μM and continued to drop very slowly to reach 1.1 μM at 30 min, still two to three times the baseline values. (C) CMBBH_2S (mean \pm SD) before the cessation of H_2S infusion as a function of the dose infused including high (H: $8.9 \pm 1.2 \mu\text{mol}/\text{min}$, black bars), moderate (M: $7.9 \pm 2 \mu\text{mol}/\text{min}$, hatched bars), and low (L: $5.1 \pm 2.4 \mu\text{mol}/\text{min}$, gray bars) then 5, 10, and 15 min into recovery. Note that there was a difference between residual levels of CMBBH_2S as a function of the dose infused. (D) Mean \pm SD value of CgH_2S before the cessation of H_2S infusion and at 3, 5, 10, and 15 min into recovery (white bars). The expected values computed based on the total time constant of the response is shown in gray bars. Note that after the cessation of H_2S exposure, CgH_2S values were indistinguishable from those due to the expected time constant of our recording setting.

As illustrated in Figure 3A, CMBBH₂S ('total' H₂S) was significantly correlated with CgH₂S. The level of CdH₂S and CMBBH₂S increased as a function of the rate of infusion but with an amount of non-soluble H₂S (difference between CdH₂S and CMBBH₂S), which reached more than 50 μM (about twice the total level of dissolved H₂S present) at the highest concentrations versus 5 μM (about six times the level of dissolved H₂S present) at the lowest levels.

As soon as H₂S infusion was stopped, expired/alveolar H₂S partial pressure dropped with kinetics (Fig. 3B) indistinguishable from the time response of our analyzers and circuit (10–12 s at a flow of 500–600 ml/min). During the entire recovery period, CgH₂S was undetectable.

CMBBH₂S determined at 1 min dropped to a level ranging from 2 to 3 μM (Fig. 3C), regardless of the level of H₂S during sulphide infusion. For the highest level of H₂S, sulphide concentrations dropped by more than 80 μM within 1 min, whereas the 50 μM of dissolved H₂S (15 μM of gaseous form) disappeared almost immediately (Fig. 3D). Following this abrupt drop, there was still a residual level of H₂S above baseline measurable by the MBB, which was found to be 3–4 times the baseline level ($0.88 \pm 0.47 \mu\text{M}$). CMBBH₂S subsided with a very slow kinetics in the 12 animals by about 1 μM/15 min, in major contrast with the very fast off kinetics during the initial first minute of recovery. Only in the animals, which received the lowest level of H₂S infusion, did CMBBH₂S return to baseline at 30 min (Fig. 3B).

Effect of Methemoglobin Infusion on the Concentration of Gaseous and Total H₂S

In a separate group of 10 rats, NaHS was infused at a rate ranging from 4.19 to 6.10 μmol/min. This caused a rise in CgH₂S to 0.34 ± 0.10 or a CdH₂S of $1.02 \pm 0.30 \mu\text{M}$ ($p < 0.001$). CMBBH₂S increased to $7.87 \pm 1.34 \mu\text{M}$ ($p < 0.01$).

As illustrated in Figure 4, MetHb infusion decreased CgH₂S by more than half, reaching 0.14 ± 0.06 (CdH₂S = $0.42 \pm 0.18 \mu\text{M}$) ($p < 0.01$). In major contrast, CMBBH₂S rose by more than seven times from $7.87 \pm 1.34 \mu\text{M}$ to $51.78 \pm 16.54 \mu\text{M}$ ($p < 0.01$; Figs. 1–3). The kinetics of reduction of CgH₂S in response to MetHb injection was very rapid with an exponential-like pattern. After correcting for the time constant of the circuit and analyzer, the response time constant following the onset of MetHb infusion was found to average 40 ± 21 s. Because the infusion of 600 mg of MetHb lasted about 1 min, less than half of MetHb was infused when CdH₂S had already decreased by more than half. After a 600 mg MetHb infusion, the level of MetHb in the arterial blood increased to $8.02 \pm 1.44\%$ ($p < 0.001$) with a Hb concentration of 13.1 ± 1.5 g/100 ml.

Three rats that received MetHb infusion during NaHS infusion (4.19–6.10 μmol/min) were studied following the cessation of NaHS infusion. CMBBH₂S rose from $8.5 \pm 3.61 \mu\text{M}$ (NaHS alone) to $47.8 \pm 20.7 \mu\text{M}$ during MetHb and NaHS infusion. CMBBH₂S remained elevated at 5 min ($10.2 \pm 7.2 \mu\text{M}$), 10 min ($4.3 \pm 1.8 \mu\text{M}$), and 15 min ($3.4 \pm 1 \mu\text{M}$). CMBBH₂S at 10 min was even higher than during control NaHS infusion prior to MetHb infusion.

Blood Infusion and Withdrawal Versus Methemoglobin Infusion

In a separate group of four rats, we assessed whether withdrawing and infusing whole blood, and therefore changing volemia and cardiac output, would affect CgH₂S in keeping with a 'volume effect' of the infusion of a MetHb solution. As illustrated in Figure 5, removing 2 ml of blood during a 7.6 μmol/min H₂S infusion increased CgH₂S from $0.43 \pm 0.16 \mu\text{M}$ to $0.64 \pm 0.25 \mu\text{M}$ (or CdH₂S from 1.29 ± 0.48 to $1.92 \pm 0.75 \mu\text{M}$) without reaching significance ($p = 0.07$), whereas infusing the same blood volume

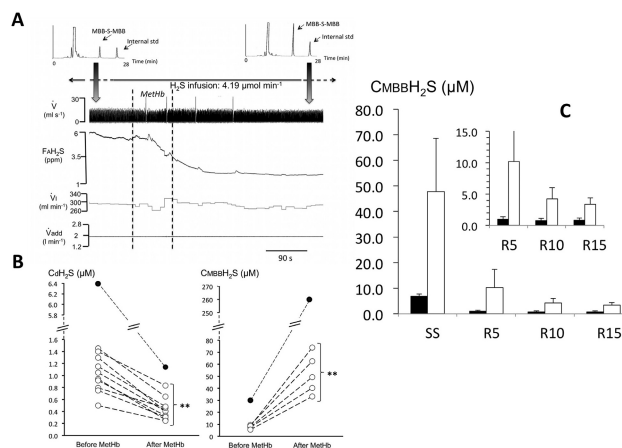


FIG. 4. (A) Recording from a rat receiving a continuous infusion of NaHS at a rate of 7.61 μmol/min. From top to bottom: respiratory flow (\dot{V}), alveolar fraction of H₂S ($F_{A\text{H}_2\text{S}}$), minute ventilation (\dot{V}_t), and additional air flow (\dot{V}_{add}) are shown. The dashed, vertical lines indicate the start and end of MetHb infusion. $F_{A\text{H}_2\text{S}}$ (see the Methods section for determination) decreased dramatically during MetHb infusion and continued to decrease beyond the period of infusion. Note that small peaks in $F_{A\text{H}_2\text{S}}$ are in each case preceded by an augmented breath (\dot{V}_t). The HPLC/fluorescence analysis of the MBB-derived internal standard and MBB-S-MBB formed from incubation of MBB reagent with the blood of the rat is depicted at the top of the trace, prior to MetHb infusion and after MetHb infusion. Note the increase in MBB-S-MBB following MetHb infusion. (B) Concentrations of dissolved H₂S (CdH₂S, left panel) and total H₂S (CMBBH₂S, right panel), before and after MetHb infusion. The concentration of the diffusible fraction of H₂S (CdH₂S) decreased significantly following MetHb infusion, whereas the non-diffusible 'sink' of H₂S (CMBBH₂S) increased following MetHb. In one rat (solid circles; not included in the statistical analysis), NaHS was infused at 15.22 μmol/min before and after MetHb. The effect of MetHb on CdH₂S and CMBBH₂S was even more striking in this animal. (C) CMBBH₂S (mean ± SD) before the cessation of H₂S infusion (SS) and during recovery from H₂S in control conditions (black bars) and following infusion of MetHb administered during sulphide infusion (open bars). Note that CMBBH₂S remains significantly higher in the tests following MetHb infusion given during recovery after H₂S than during control recovery. **Significantly different from before MetHb at $p < 0.01$.

rapidly decreased CgH₂S from $1.29 \pm 0.51 \mu\text{M}$ to $0.43 \pm 0.17 \mu\text{M}$ ($p < 0.05$). These changes in Cg/dH₂S were therefore related to the change in volemia or cardiac output caused by the infusion or the withdrawal of blood, the latter likely offering less Hb to combine with H₂S per unit of time. They were of short duration as illustrated in Figure 5. However, infusing a similar volume of MetHb in saline (300 mg, 150 mg/ml) caused a response that was different both quantitatively and qualitatively. The reduction in CgH₂S was significantly more pronounced following MetHb infusion (CgH₂S decreased from $0.84 \pm 0.24 \mu\text{M}$ to $0.28 \pm 0.08 \mu\text{M}$). In addition, the most remarkable effect of the presence of MetHb was that further withdrawal or infusion of blood had only trivial effects on CgH₂S (0.34 ± 0.14 and $0.28 \pm 0.09 \mu\text{M}$ respectively, not significantly different from each other [$p = 0.88$]). In some cases, these changes were undetectable.

CgH₂S and CMBBH₂S During and Following NaHS Infusion in the Sheep

Figure 6 is an illustration of the change in the alveolar fraction of H₂S (gaseous) at various rates of NaHS infusion in one animal, along with the averaged response (Fig. 7A). H₂S was undetectable in its soluble form in baseline conditions whereas baseline CMBBH₂S was found to average $0.61 \pm 0.28 \mu\text{M}$. The relationship between CgH₂S, CMBBH₂S, and the rates of infusion is displayed in Figure 7C along with the levels of H₂S at which breathing was stimulated and an apnea occurred: breathing was

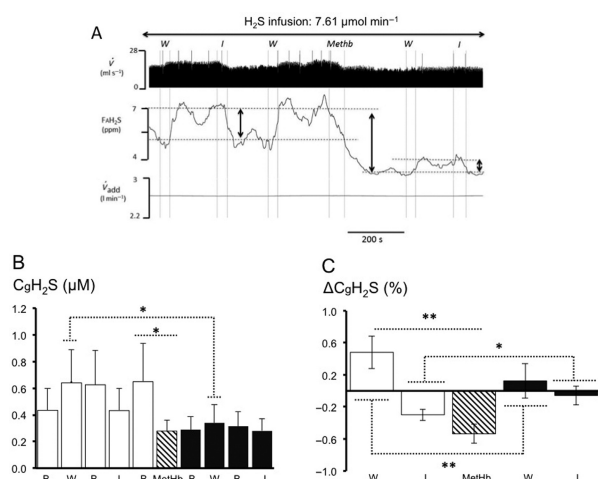


FIG. 5. Effects of an acute change in volemia on CgH_2S . (A) Recording during withdrawal (W) or infusion (I) of 2 ml of blood before and after infusing 2 ml of MetHb solution. Respiratory flow (\dot{V}), alveolar fraction of H_2S (FAH_2S), and additional air flow (\dot{V}_{add}) signals are shown. (B) Effect of blood versus MetHb infusion on mean CgH_2S , the symbol B related the baseline values before withdrawal (W) or infusion (I) of blood. (C) Change in CgH_2S after withdrawal or infusion of blood or MetHb compared with values obtained before each treatment. Withdrawing and infusing blood produced a small rise and decrease in CdH_2S , respectively. Infusing MetHb, instead of blood, produced a dramatic decrease in CgH_2S ($p < 0.01$) that persisted, and in most cases prevented, any other effects of volemia on CgH_2S . *Significantly different at $p < 0.05$, **Significantly different at $p < 0.01$.

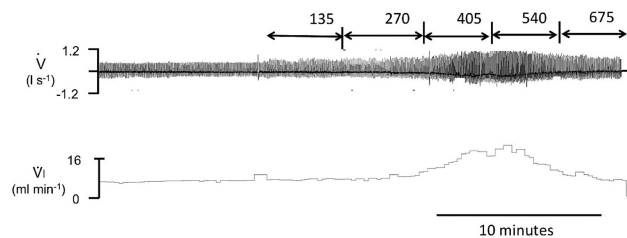


FIG. 6. Example of the effects of infusing NaHS on breathing in an adult sheep at incremental levels. Note that in this example, breathing started to rise between an infusion rate of 270 and 405 $\mu\text{mol}/\text{min}$ whereas an apnea occurred at 675 $\mu\text{mol}/\text{min}$.

stimulated at an infusion rate of 270 $\mu\text{mol}/\text{min}$, leading to values of CgH_2S , CdH_2S , and $CMBBH_2S$ that averaged 0.07 ± 0.03 , 0.21 ± 0.90 , and $9.59 \pm 3.53 \mu\text{M}$, respectively. A terminal apnea occurred at CgH_2S , CdH_2S , and $CMBBH_2S$ concentrations, which averaged 4.93 ± 2.78 , 14.79 ± 8.34 , and $106.69 \pm 22.99 \mu\text{M}$, respectively. The rates of infusion leading to hyperventilation or to apnea were very close, resulting in a very steep dose-toxic effect relationship as soon as symptoms occurred (Fig. 7C). The relationship between CgH_2S and $CMBBH_2S$ revealed that the ability of the blood to combine with H_2S was much larger in the rat at any given level of CgH_2S or infusion rate. Another way to express this notion is illustrated in Figure 8; the concentration of sulphide as a function of the rate of infusion per body weight was higher in the combined form but not in the soluble form in the sheep when compared with the rat.

At the cessation of NaHS infusion, CgH_2S dropped with virtually no delay, the kinetics of this response was indiscernible from the off-transient of the analyzer, like in the rat. $CMBBH_2S$ also dropped very rapidly reaching a residual level of about $2 \mu\text{M}$ at 5, 10, and 15 min but remained significantly higher than baseline values (Fig. 7C).

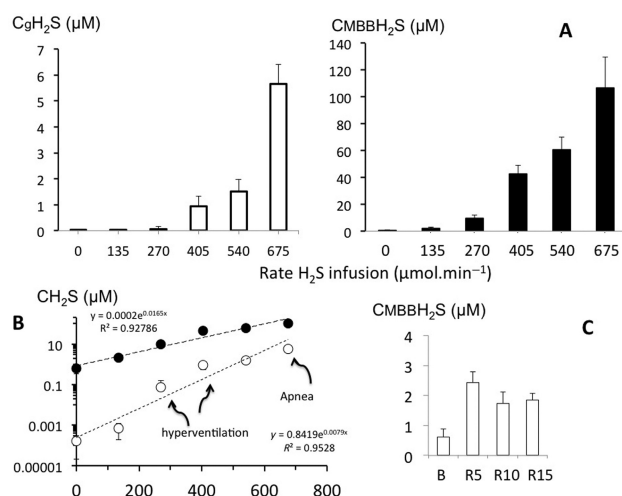


FIG. 7. (A) Concentration (mean \pm SD) of the gaseous form of H_2S in the arterial blood (CgH_2S ; open bars) and of H_2S measured in the blood using MBB ($CMBBH_2S$; black bars). Increasing the rate of NaHS infusion caused an increase in CgH_2S and $CMBBH_2S$. The majority of H_2S found in the blood was not in the dissolved form. CgH_2S increased from zero to a value of $5.6 \mu\text{M}$ when a terminal apnea occurred, corresponding to $CMBBH_2S$ that averaged $106.8 \mu\text{M}$, up to 20 times the gaseous form. As shown in (B), the H_2S concentration increased exponentially with the rate of infusion and the ratio between CgH_2S (open circles) and $CMBBH_2S$ (black circles) decreased as H_2S concentrations increased. (C) $CMBBH_2S$ in baseline conditions and following H_2S infusion at 5, 10, and 15 min into recovery; note that $CMBBH_2S$ dropped dramatically after H_2S infusion but remained higher than baseline for at least 15 min.

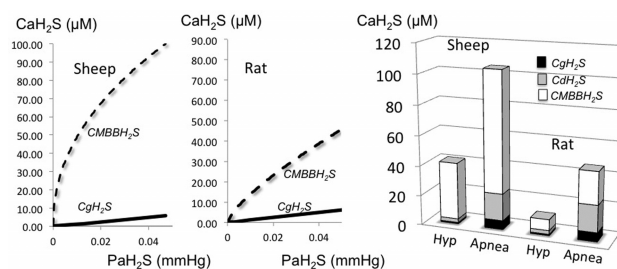


FIG. 8. Relationship between partial pressure of H_2S and the various pools of H_2S in the arterial blood during H_2S exposure in the sheep and the rat. These graphs have been created based on the exponential regressions obtained in the current and previous studies on the rat and the present results for the sheep. Note that the ability of the blood to transport H_2S is much higher in the sheep than in the rat. In the left-hand panel, the values of CgH_2S , CdH_2S , and $CMBBH_2S$ for the sheep and the rat are presented at levels of infusion wherein hyperventilation (Hyp) and apnea occurred. Note that the major part of H_2S in the blood is not in gaseous form and that the ratio between CgH_2S and $CMBBH_2S$ decreases as the level of H_2S increases.

Effects of Hydroxocobalamin and Methemoglobin in the Sheep

HyCo (70 mg/kg in 200 ml) was injected over a 5 min period in each sheep whereas NaHS infusion (270–405 $\mu\text{mol}/\text{min}$) was maintained for at least 15 additional min. As illustrated in Figure 9A in one animal and in Figure 9C for the average response, HyCo infusion decreased CgH_2S from an average of $1.01 \pm 0.4 \mu\text{M}$ to $0.07 \pm 0.09 \mu\text{M}$ within a few minutes ($p < 0.01$). In contrast, $CMBBH_2S$ did not change significantly (37.2 ± 13.1 vs. $26.1 \pm 14.9 \mu\text{M}$). The kinetics of the reduction of CgH_2S in response to HyCo injection was very rapid (Fig. 9A). CgH_2S remained low for

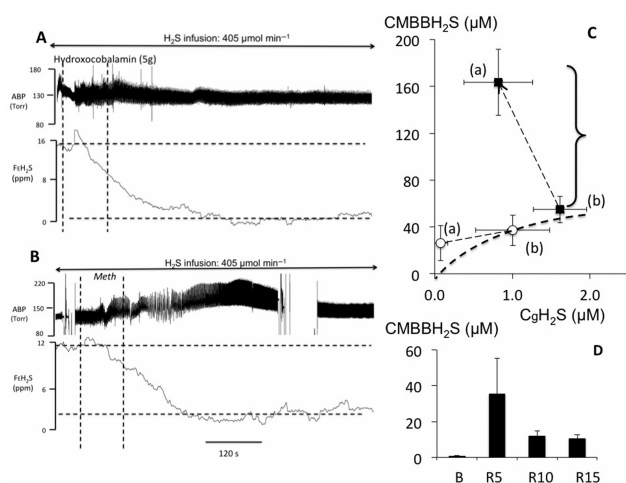


FIG. 9. (A) Recording obtained in one sheep receiving a continuous infusion of NaHS at a rate of 405 $\mu\text{mol}/\text{min}$. From top to bottom: systemic blood pressure and mixed expired fraction of H_2S are shown. The dashed, vertical lines indicate the start and end of HyCo solution injection. The gaseous form of H_2S in the blood decreased dramatically during HyCo infusion reaching almost zero despite the persistence of sulphide infusion. (B) Recording obtained in one sheep receiving a continuous infusion of NaHS at a rate of 405 $\mu\text{mol}/\text{min}$, same traces as (A). The two dashed, vertical lines indicate the start and end of MetHb infusion. $\text{F}_i\text{H}_2\text{S}$ decreased dramatically during MetHb infusion and continued to decrease beyond the period of MetHb infusion. Note the increase in ABP that was systematically observed in each test following MetHb injection. (C) Mean \pm SD relationship between the concentrations of gaseous H_2S (CgH_2S) and total CMBBH₂S, before (b) and after (a) MetHb infusion (black symbols) and HyCo infusion (open symbols). CgH_2S significantly decreased following MetHb infusion just like in the rat with a fourfold increase in CMBBH₂S associated with an increase of MetHb to $6.34 \pm 0.73\%$. The decrease in CgH_2S was even more significant following HyCo infusion with a small decrease in CMBBH₂S, suggesting a different mode of action of these two antidotes on the pools of combined H_2S . (D) CMBBH₂S (mean \pm SD) before H_2S infusion (baseline) and during recovery from H_2S following infusion of MetHb administered during sulphide infusion. Note that CMBBH₂S is 15–20 times higher in the tests following MetHb infusion given during recovery from H_2S infusion than during control recovery as shown in Figure 7.

10–12 min during the infusion, and then increased returning to pre-Hyco levels within 20 min.

Infusion of MetHb lasted about 2–3 min. Like in the rat, CgH_2S decreased by half from 1.61 ± 0.34 to $0.83 \pm 0.44 \mu\text{M}$ ($p < 0.01$), whereas CMBBH₂S rose by more than threefold, from 54.9 ± 11.4 to $163 \pm 28.1 \mu\text{M}$ ($p < 0.01$) as illustrated in Figures 9B and 9C. The level of MetHb in the arterial blood increased to $6.34 \pm 0.73\%$ ($p < 0.001$). In four of the five sheep, CMBBH₂S was measured following the cessation of NaHS infusion (after having received a MetHb infusion) at 5, 10, and 15 min. As shown in Figure 9D, CMBBH₂S remained much higher than during control recovery, suggesting that H_2S combined to ferric iron remains present in the blood following the cessation of sulphide exposure.

DISCUSSION

We found that, in the rat, infusion of NaHS at a constant rate ranging from 4 to 9 $\mu\text{mol}/\text{min}$ produced an increase in the concentration of dissolved and combined H_2S , similar to those we previously obtained during a ramp-like increase in H_2S in the same species (Klingerman et al., 2013). At the lowest rate of infusion, CgH_2S and CdH_2S represent a small portion of the total pool of H_2S present in the blood as measured by the MBB method (Fig. 8). This proportion increased as the rate of infusion and CgH_2S

concentrations increased. At the cessation of sulphide exposure, dissolved H_2S dropped with virtually no delay to undetectable levels, whatever the rate of infusion of H_2S , whereas CMBBH₂S dropped to levels ranging from 2 to 3 μM in less than 1 min, but remained higher than baseline for at least 30 min. Very similar results were obtained in the sheep. MetHb infusion increased dramatically the ability of the blood to combine with free H_2S , reducing, in turn, CgH_2S . Finally, HyCo decreased the concentration of the gaseous form of H_2S to almost zero and thus displaced H_2S combined with the Hb, but for a limited period of time if infusion is maintained. These results and their implications are discussed in the following paragraphs.

Pools of H_2S and Their Kinetics in the Rat and Sheep During and Following H_2S Exposure

Based on the methodology used to measure H_2S , one can distinguish, for the sake of convenience, different pools of H_2S in the blood. First, there is a gaseous pool which diffusion and rate of exchange can be understood just like for any other gas; however, due to the extremely high solubility of H_2S , compared with O_2 or even CO_2 , very small partial pressure can maintain a fair amount of H_2S allowing diffusion to persist. By comparison, a partial pressure of about 0.01 Torr is required to maintain a concentration of about 0.01 μM of dissolved O_2 , whereas a partial pressure of only 0.0001 Torr can achieve the same effects for H_2S . We found that in both rats and sheep, these concentrations represent a small portion of the total H_2S present in the blood (Fig. 8). The second component is represented by the anion HS^- , as for every molecule of H_2S in gaseous form, 3–4 molecules of HS^- are present at physiological pH (Almgren et al., 1976; Millero, 1986), increasing the capacity of the plasma to transport H_2S by 3–4 times at any given H_2S partial pressure. These two components represent the soluble/dissolved pool of sulphide. The second pool is to be accounted for by the discrepancy between the concentration of the pool of exchangeable sulphide—measured based on expired gas or by amperometry (Wintner et al., 2010)—and the total amount of H_2S in the blood measured by the MBB technique (Klingerman et al., 2013; Wintner et al., 2010). This difference is likely due to the presence of a pool of H_2S combined with Hb and plasma proteins. Increasing the level of MetHb in the blood dramatically increased CMBBH₂S.

The concentrations of gaseous H_2S required to stimulate the chemoreceptors, or stop breathing in the sheep, were not very different from the level required to produce symptoms in the rat (Klingerman et al., 2013) (Fig. 8). Indeed, despite a weight of about 100 times larger than the rat, any given rate of infusion/kg led to similar levels of CgH_2S that were previously found in the rat when hyperventilation or apnea was produced (Klingerman et al., 2013), i.e., less than 1 μM for breathing stimulation and around 5–8 μM for apnea. The only difference found in the sheep was a higher concentration of total H_2S for any given level of gaseous H_2S as compared with levels in the rat (about 2 \times). It is quite intriguing that the same rate of H_2S infused per kilogram produced the same effects and led to similar concentrations of gaseous H_2S in both species, whereas we would have anticipated that a much lower rate of H_2S would produce greater effects in larger mammals. This suggests that the rate of disappearance of the dissolved form of H_2S , per gram of tissue, is very similar in these two species. As CdH_2S remains stable throughout a period of several minutes of infusion at a rate of 8–12 $\mu\text{mol}/\text{kg}/\text{min}$, both in the rat and in the sheep, the rate of elimination/oxidation of H_2S must be at least 8–12 $\mu\text{mol}/\text{kg}/\text{min}$. This level of oxidation is perfectly compatible with the disappearance of H_2S during recovery, wherein for

a concentration of 80 μM of CMBBH₂S, 6 μM of CgH₂S vanishes within a minute. Indeed, it did not take more than 1 min for gaseous H₂S present at these lethal concentrations to disappear. Incidentally, the rate of elimination of H₂S by the lungs represents only a small proportion of the rate of H₂S infused in both species (Klingerman et al., 2013). CMBBH₂S values obtained in humans during low levels of sulphide infusion (Toombs et al., 2010) fit with the effects observed in our sheep and suggest that large mammals including humans may possess a higher ability to combine H₂S than the rat.

The most intriguing observation is certainly the baseline, as well as the persistence of a very small pool of H₂S in the blood with a very slow time constant. The levels of CMBBH₂S, both in the baseline condition and during recovery, were indistinguishable between the sheep and the rat. H₂S fixed on the Hb or proteins certainly accounts for this small H₂S pool that is not rapidly exchangeable. The much greater level of CMBBH₂S after MetHb infusion supports this contention. This also suggests that H₂S inside the cells may also remain combined with other metallo-compounds (Haouzi and Klingerman, 2013), the main “targets” of sulphide toxicity, beyond the period of exposure. Similarly, H₂S combined with cysteine residues, if part of this pool in the intracellular compartment, would be likely to persist as well (Mustafa et al., 2009; Warenycia et al., 1990).

Methemoglobin Solution and Hydroxocobalamin as Antidotes During H₂S Poisoning

As presented in the Introduction section, various agents have been proposed to treat H₂S poisoning: hyperoxia (Bitterman et al., 1986; Haouzi et al., 2011a; Smilkstein et al., 1985, 1976), metallo-compounds (ferric iron using nitrite-induced methemoglobinemia or cobalt in vitamin B12) (Haouzi et al., 2011b; Smith, 1969; Smith et al., 1976; Truong et al., 2007; Van de Louw and Haouzi, 2012), reducing agents (Warenycia et al., 1990), sodium bicarbonate, or high level of glucose (Almeida and Guidotti, 1999; Guidotti, 2010).

Sodium nitrite-induced methemoglobinemia has long been proposed as a treatment of H₂S poisoning in humans (Hall and Rumack, 1997; Smith, 1981; Smith and Gosselin, 1979); animal studies have demonstrated a protective effect against H₂S-induced lethality in rodents when injected “prior” to the toxic exposure (Smith and Gosselin, 1979; Smith et al., 1976). The oxidation of the ferrous (Fe²⁺) to ferric iron (Fe³⁺) present in Hb, produced by sodium nitrite, allows the combination of H₂S into a non-soluble, i.e., non-diffusible, form (Smith and Gosselin, 1966) and seems also to be able to catalyze the oxidation of H₂S (Beck et al., 1981).

Twenty milligram per kilogram of sodium nitrite (Kohn et al., 2002) has been previously used intravenously, resulting in the production of 10–25% MetHb 20 min after injection (Kohn et al., 2002). We have recently shown that much lower levels of nitrite (5 mg/kg) and MetHb (3%) were sufficient to prevent the ventilatory stimulation produced by an IV bolus of H₂S (Van de Louw and Haouzi, 2012). Twenty minutes following the injection of low levels of sodium nitrite, we found that the response to a bolus injection of a 2.4 mM (0.5 ml) solution of H₂S was virtually abolished (Van de Louw and Haouzi, 2012). It has been proposed that 2–4 moles of sulphide can be inactivated *in vivo* for each available ferric heme group in MetHb (Smith, 1967). Kohn and colleagues have reported that 30 min were needed to maximize MetHb concentrations in the blood after IV infusion of 20 mg/kg of nitrite to rats (Kohn et al., 2002). Besides its delay of action, the major limitations of using nitrite-induced methemoglobinemia to counteract H₂S intoxication are (1) the deleterious effects

of nitrite-induced vasodilation (Hall et al., 1989), (2) the reduction in the amount of functional Hb and thus a reduction in O₂ transport, and (3) the negative interaction between nitrite and O₂ in the production of MetHb (Beck et al., 1981). These limitations could be overcome by the use of a solution of free MetHb. The lack of reductase in a free Hb solution, normally present in red cells, allows the spontaneous oxidation of ferrous iron into stable MetHb. We took advantage of this usually undesirable natural oxidation to produce a solution of crystalline Hb containing 98% of MetHb. Rapid infusion of such a solution was able to increase the MetHb level to 8% and to dramatically reduce gaseous H₂S concentrations in the blood via a rise in the CMBBH₂S sink by more than seven times during sub-lethal infusion of H₂S. A similar effect could be produced using a much lower level of MetHb as suggested by the kinetics of the response, wherein CgH₂S dropped whereas only a small portion of total MetHb had been infused. These effects, which remained present following H₂S exposure although MetHb was injected during H₂S infusion, were similar in the rat and in the sheep.

There was a large discrepancy between the decrease in the CgH₂S, which dropped by half (and by less than 1 μM), and the rise in CMBBH₂S, which increased by more than 40 μM . This suggests that not only a very large portion of the soluble form of H₂S (CgH₂S) was combined with MetHb, but also H₂S that should have diffused into the tissues and been oxidized was trapped in the process. There are clinical reports supporting the use of nitrite-induced methemoglobinemia after H₂S intoxication (Hall and Rumack, 1997). For obvious reasons, these observations remain anecdotal, as control case studies in humans cannot be performed.

The combination of H₂S and its “transporter,” ferric iron molecules, was almost immediate. Changing the cardiac output by withdrawing and infusing 2 ml of blood affected the concentration of dissolved H₂S with an inverse relationship. This suggests that the level of Hb available to interact with H₂S per unit of time does influence the ratio between combined and dissolved H₂S. The rate of complexation of H₂S in the blood is in part controlled by the rate at which Hb circulates. This phenomenon is therefore magnified dramatically by the presence of MetHb that abolished the volemia-induced CgH₂S changes. In other words, the infusion of MetHb not only had a persistent effect on CgH₂S, but the presence of MetHb made the ability of the blood to combine with H₂S almost independent of volemia/cardiac output.

Large doses of HyCo have also been shown to reduce the hepatotoxicity of H₂S in a rat model, prevent H₂S-induced mortality in mice (Mihajlovic, 1999; Truong et al., 2007), and magnify the ability of the blood or tissue of rats exposed to HyCo to oxidize hydrogen sulphide (Van de Louw and Haouzi, 2012). In addition, vitamin B12 is currently used as an antidote for cyanide intoxication at a dose 10⁶ times the normal daily intake (Fortin et al., 2006; Hall et al., 2009; Shepherd and Velez, 2008). At this dose (5 g in adult), concentrations of vitamin B12 in the blood increase from a few pM to several mM range in humans (Forsyth et al., 1993) and more than 500 μM in the rat. In contrast to MetHb, which remains in the blood, vitamin B12 at this concentration diffuses into the cells and into the mitochondria via non-transcobalamin mechanisms (Gimpert et al., 1975; Hall et al., 1984), where cobalt could complex H₂S and catalyze its oxidation. We found that vitamin B12 injection during H₂S infusion in the sheep was able to drop the level of dissolved H₂S with a much larger magnitude than MetHb, whereas total H₂S was much higher than expected from the reduction in soluble H₂S. As CMBBH₂S follows the drop in CgH₂S during recovery, CMBBH₂S seems to constitute, except for few residual μM , a pool

exchangeable almost immediately with the diffusible part. This would suggest that H₂S combined with HyCo is accessible to the MBB measurement and may be able to displace H₂S fixed on Hb.

Of note is that, to reduce the number of sheep used in the study, the infusion of MetHb was performed in animals that had previously received HyCo. The effects of MetHb were tested when the effects of Hyco had already vanished at least on sulphide concentrations; still, results were identical to the rat.

These two antidotes had a very rapid effect on H₂S concentrations, occurring within tens of seconds, but seem to act against H₂S poisoning very differently on the various pools of H₂S and thus may have a very different impact on what could have happened whenever injected “after” exposure. Indeed, the putative efficacy of an antidote to be used when a patient is withdrawn from the source of H₂S intoxication relies on two main prerequisites: (1) H₂S must still be present in the body in a form that contributes, per se, to the persistence of toxic effects following H₂S exposure, and (2) the recovery kinetics of the different forms of H₂S in the blood and tissues must be compatible with a timeline allowing rescuers to administer an antidote. In other words, if all gaseous H₂S is already gone, the very reason for using an antidote becomes obsolete. Although gaseous H₂S is probably not present for more than few minutes, at best, after the cessation of exposure, there is still no clear answer about how long non-soluble forms of H₂S, such as with Cu, Zn, Fe, or Co containing metallo-proteins or sulfane sulphur, could persist. This makes the use of MetHb, which remains in the intravascular compartment and which acts by increasing the H₂S sink in the blood, an approach that might be very limited at a time when all dissolved H₂S has already been oxidized or eliminated. A similar reasoning can be applied for vitamin B12. The only difference with MetHb is that vitamin B12 can diffuse into the cells and into the mitochondria via non-transcobalamin mechanisms (Gimpert *et al.*, 1975; Hall *et al.*, 1984). Whether cobalt compounds can interact with the form of H₂S combined with metallo-proteins and displace some of the H₂S present on cytochrome c while no free H₂S is present in the blood and in the tissues remains an outstanding question.

Finally, it is possible to imagine clinical scenarios wherein the fast kinetics of gaseous sulphide recovery could be slowed, during a very severe reduction in cardiac output, wherein the rate of pulmonary exchange is decreased. A patient is found in cardiac arrest or in extremely severe shock, could present such a phenomenon. Nevertheless, we found that the fast disappearance of the soluble form of H₂S is still present in the most severe forms of intoxication, wherein blood pressure was reduced. The extreme diffusibility of H₂S in its gaseous form will also allow the H₂S concentration to drop in the alveolar gas at a very fast rate, even during an apnea, as long as some cardiac output/pulmonary blood flow is maintained whether H₂S is inhaled instead of infused. For all practical purposes, one could speculate that any improvement of hemodynamics should by itself improve the ability of H₂S to be exchanged and oxidized in the tissues, a strategy that may prove to be even more effective than using any trapping molecules. Of note is that the blood and the plasma are able to continue to oxidize H₂S at a very fast rate (Klingerman *et al.*, 2013; Olson, 2011a; Wintner *et al.*, 2010).

In conclusion, H₂S metabolism during H₂S administration is very similar in a large and a small mammal. H₂S presence in its free, soluble form cannot be sustained in a durable manner in the blood and tissues beyond a minute in both species even at levels producing a terminal apnea. Whereas MetHb and HyCo solutions are very potent antidotes against H₂S, the efficacy of administering metallo-compounds following H₂S exposure can

be challenged on the basis of their interaction with free H₂S and kinetics described in both animal models.

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