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Passive loss of hydrogen sulfide in biological experiments

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ABSTRACT

Hydrogen sulfide (H_2S) is a volatile gas of considerable interest as a physiologically relevant signaling molecule, but this volatility has typically been overlooked in the context of biological experiments. We examined volatility of 10 and 100 μM H_2S ($Na_2S{\cdot}9H_2O$) in real time with polarographic electrodes in three commonly employed experimental apparatuses: 24-well tissue culture plates (WP), muscle myograph baths (MB), and the Langendorff perfused heart apparatus (LPH). H_2S loss from all apparatuses was rapid and exponential, with half-times ($t_{1/2}$) of 5 min (WP), less than 4 min (MB), and less than 0.5 min (LPH). The $t_{1/2}$ for H_2S loss from MB bubbled with 100% oxygen was slightly longer than that for MB bubbled with 100% nitrogen; both were significantly shorter than stirred but unbubbled MB (>9 min). Therefore, even without tissue, H_2S rapidly disappears from buffer under a variety of experimental conditions, and this is due to volatilization, not oxidation. The inability to maintain H_2S concentration, even briefly, questions the accuracy of dose–response studies and the relevance of long-term (>10 min) exposure to a single treatment of H_2S. These results also help to explain the discrepancy between low H_2S concentrations in blood and tissues versus high concentrations of exogenous H_2S required to produce physiological responses.

Hydrogen sulfide (H_2S)[1] produces physiopharmacological responses in virtually every organ system [1–9], and it has been proposed to serve as a signaling "gasotransmitter" along with nitric oxide and carbon monoxide [10]. Heightened interest in the biological actions and therapeutic potential of H_2S has been accompanied by reevaluation and refinement of methods used to measure H_2S in blood and tissues. Recent studies have provided evidence that the concentrations of H_2S in both blood [11] and tissues [12–14] are considerably lower than previously thought, and they are also ten- to several hundred-fold lower than the concentrations of exogenous H_2S necessary to initiate biological responses [15].

There are three possible explanations for the considerable disparity between H_2S concentrations measured in tissues and blood and those required to initiate biological responses. First, some of the reported effects of H_2S could be pathophysiological, not physiological. At high concentrations, H_2S could be a significant reductant; above 40 μM, H_2S can inhibit cytochrome *c* oxidase [16,17], and salts commonly used to prepare H_2S (i.e., sodium hydrosulfide [NaHS]) are alkaline if unbuffered [18]. Second, it is possible that exogenously applied H_2S is rapidly consumed/metabolized by tissues, which would necessitate an excessive supply of exogenous H_2S to minimally alter intracellular H_2S concentration. In fact, both passive and active mechanisms of H_2S oxidation have been demonstrated. Chen and Morris [19] showed that H_2S is spontaneously oxidized in the presence of metal catalysts, and Lagoutte and coworkers [20] and Olson and coworkers [21] showed that H_2S is rapidly oxidized by biological tissues under normoxic conditions. Third, because H_2S is a gas, volatilization of H_2S from the surface of tissue baths or culture media could also affect the H_2S concentration. This could be especially problematic in experiments that are bubbled with oxygen or other gas mixtures or in experiments where the surface area is large relative to the volume of incubation medium or buffer. Unfortunately, these conditions are encountered in practically all biological experiments. To our knowledge, the last possibility, passive volatilization of H_2S from tissue media or buffers during an experiment, has not been critically examined. This is especially surprising given the well-known odor of rotten eggs that appears immediately on solvation of sulfide salts. H_2S volatility is also the basis for several methods commonly used to measure the concentration of H_2S in tissues or the rate of tissue H_2S production; H_2S equilibrium into headspace gas has been measured by gas chromatography, and H_2S absorbed onto zinc acetate-soaked filter paper suspended above tissue has been measured with the methylene blue method [15].

The effects of H_2S on the cardiovascular system is arguably the most intensely studied area of H_2S biology [4]. Accordingly, we

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[1] *Abbreviations used:* H_2S, hydrogen sulfide; NaHS, sodium hydrosulfide.

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chose the three methods most commonly used in these studies—standard tissue culture well plates, muscle tissue baths (myographs), and the Langendorff perfused heart apparatus—to examine H_2S volatilization in the absence of tissue. H_2S was measured with a polarographic (amperometric) electrode, which permits an assessment of H_2S gas in real time and on unadulterated samples [22,23]. The intent of these studies was to determine whether the experimental apparatus and protocol could affect H_2S concentration independent of tissue activity. Accordingly, the experiments were designed to replicate the above experimental conditions as closely as possible in the absence of tissue.

Materials and methods

H_2S measurement

An amperometric H_2S sensor was built in-house [11] or purchased (model HS-700, Innovative Instruments, Tampa, FL, USA) and used in conjunction with an Apollo free radical analyzer (World Precision Instruments, Sarasota, FL, USA). These sensors measure H_2S gas that is in the equilibrium: $H_2S \rightleftarrows HS^- \rightleftarrows S^{2-}$, with $pKa_1 = 6.9$ and $pKa_2 > 11$ [24]. At physiological pH (~7.4 blood and ~6.9 intracellular), the concentration of H_2S ranges from 25% to 50% of the total dissolved sulfide. However, the equilibrium can be displaced by H_2S diffusion out of the aqueous phase, and this can continue until the added sulfide is exhausted. Because the sensor measures H_2S gas in real time in biological fluids, it is an ideal reporter of H_2S volatilization. The sensor was calibrated with dissolved buffered $Na_2S \cdot 9H_2O$ (hereafter referred to as Na_2S) prior to or after every experiment, and sensor current was recorded continuously. By convention, H_2S refers to the polarographic measurement of H_2S gas in total dissolved sulfide ($H_2S + HS^- + S^{2-}$), with the latter derived from the molar concentration of dissolved Na_2S. All experiments were performed at room temperature.

H_2S formation on solvation

A 2.4-mg crystal of Na_2S was added to 100 ml of Hepes buffer (final theoretical concentration = 100 μM) in a 150-ml Pyrex beaker with continuous stirring. The crystal was immediately dissolved, and the response was measured for 20 min.

H_2S loss from cell culture well plates

Na_2S sufficient to produce a theoretical concentration of either 10 or 100 μM dissolved H_2S was added to 1.5 ml of Hepes buffer in open, flat-bottom, 24-well polystyrene Linbro tissue culture plates (Hampton Research, Aliso Viejo, CA, USA). The samples were gently stirred, and H_2S concentration was measured for 15–20 min. To verify that the majority of H_2S lost from the buffer was due to volatilization and not absorption by the polystyrene, the wells were covered in a second series of experiments with a 2-cm-thick piece of Plexiglass with a hole in the center through which the H_2S sensor was inserted. The headspace gas was eliminated by ensuring that the wells were filled to the level of the cover.

H_2S loss from tissue myograph baths

The first series of experiments examined the rate of H_2S loss from unbubbled myograph baths. Hepes buffer (5 ml) was placed in a standard 6-ml water-jacketed myograph tissue bath (Radnoti, Monrovia, CA, USA) containing a cylindrical stir bar for slow agitation. Dissolved Na_2S was added to produce a final concentration of 10 and 100 μM. H_2S concentration was measured for 30–60 min.

The second series of experiments examined the effect of bubbling on volatilization of H_2S. The stir bar was removed, and the buffer was bubbled with either 100% nitrogen (N_2) or 100% oxygen (O_2) gas through a glass frit in the myograph. Gas flow was measured on a flow meter (Porter Instrument, Hatfield, PA, USA) and maintained at 8.4 cc/min. Dissolved Na_2S was added to produce a final theoretical concentration of 10 and 100 μM. The experiment was terminated when more than 90% of the H_2S was lost, usually within 15 min.

H_2S loss from Langendorff isolated heart apparatus

A modified Langdendorff perfused heart apparatus was used to examine the possibility of H_2S loss due to volatilization during isolated heart experiments. A water-jacketed 60-cm-high distillation chamber was primed with 30 ml of Hepes buffer, and a peristaltic pump circulated the buffer through the chamber and into a 5-ml beaker suspended beneath the upper stopper. The H_2S sensor was placed in this beaker. H_2S sufficient to increase the buffer concentration to 10 and 100 μM was injected via a stopcock into the outflow tract from the pump and allowed to recirculate at a rate of 30 ml/min. H_2S concentration was measured for 10 min.

H_2S injected into the supply line produced a very intense and transient response that rapidly disappeared. A second small peak was observed approximately 1 min later, presumably due to first-pass recirculation of the bolus. The concentration of H_2S slowly declined after the second peak. Because this second peak prevented a two-component analysis of the total decay curve, the first and second curves were analyzed separately as one-component systems. The odor of H_2S was also noticed when the apparatus was opened. Because this might decrease the rate of volatilization due to back flux of H_2S from the airspace, a second set of experiments was performed, where a needle was inserted through the lower stopper and 100% N_2 was flushed through the apparatus and out the upper stopper. A second recirculation peak was not noticed in these experiments, which permitted a two-component analysis.

Data analysis

H_2S concentration was recorded as current (in nA) and copied into an Excel spreadsheet for further analysis. The data were analyzed using one- or two-component exponential curve-fitting software with Table Curve (Jandel, Chicago, IL, USA). The rate of H_2S loss from the well plates was usually best fit by a mono-exponential decay curve of the following type: $H_2S_t = H_2S_0 e^{-kt}$, where H_2S_t is the concentration of H_2S at time t, H_2S_0 is the initial H_2S concentration, e is the natural log, and k is the rate constant (i.e., the fractional loss of H_2S per minute). The half-time ($t_{1/2}$) is the time it takes the H_2S to be halved and is calculated from the rate constant: $t_{1/2} = 0.693/k$. This equation characterizes simple mixing into, or in our case diffusion out of, a single volume (compartment). The rate of 10 μM H_2S loss from the myograph baths was also most often fit best by the mono-exponential decay curve. However, the rate of 100 μM H_2S loss from the myograph bath was often fit with a two-component exponential decay curve of the following type: $H_2S_t = H_2S_1 e^{-k1t} + H_2S_2 e^{-k2t}$, where H_2S_1 and H_2S_2 are the two components, $k1$ and $k2$ are the respective rate constants, and from them are calculated the two half-times, $t_{1/2}1$ (the fast component) and $t_{1/2}2$ (the slow component). A two-compartment model indicates that there are two events occurring simultaneously and that the rates ($t_{1/2}$ and k) at which each event occurs are different. The two-compartment model also allows one to identify the $t_{1/2}$ and k for both processes. When possible, data for both one- and two-component analysis are included in the myograph bath results. The initial and recirculation peaks observed with the closed Langendorff apparatus were analyzed as separate one-component