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Working with "H_2S": Facts and apparent artifacts

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ABSTRACT

Hydrogen sulfide (H_2S) is an important signaling molecule with physiological endpoints similar to those of nitric oxide (NO). Growing interest in its physiological roles and pharmacological potential has led to large sets of contradictory data. The principle cause of these discrepancies can be the common neglect of some of the basic H_2S chemistry. This study investigates how the experimental outcome when working with H_2S depends on its source and dose and the methodology employed. We show that commercially available NaHS should be avoided and that traces of metal ions should be removed because these can reduce intramolecular disulfides and change protein structure. Furthermore, high H_2S concentrations may lead to a complete inhibition of cell respiration, mitochondrial membrane potential depolarization and superoxide generation, which should be considered when discussing the biological effects observed upon treatment with high concentrations of H_2S. In addition, we provide chemical evidence that H_2S can directly react with superoxide. H_2S is also capable of reducing cytochrome c^{3+} with the concomitant formation of superoxide. H_2S does not directly react with nitrite but with NO electrodes that detect H_2S. In addition, H_2S interferes with the Griess reaction and should therefore be removed from the solution by Cd^{2+} or Zn^{2+} precipitation prior to nitrite quantification. 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) is reduced by H_2S, and its use should be avoided in combination with H_2S. All these constraints must be taken into account when working with H_2S to ensure valid data.

1. Introduction

The role of hydrogen sulfide (H_2S) as a biologically relevant gaseous signaling molecule began to emerge when the seminal discovery was made that H_2S could serve as an endogenous neuromodulator [1]. Almost two decades later, H_2S is suggested to be the third gasotransmitter, together with nitric oxide (NO) and carbon monoxide (CO) [2–5]. Several important physiological functions have been shown to be regulated by H_2S (including neuromodulation, regulation of blood pressure and immunomodulation) [1,6–10]. In addition, H_2S has strong pharmacological effects (in particular, in preventing ischemia–reperfusion injury and in inducing a suspended animation-like state in animals) [11–14]. These properties have led to increasing numbers of studies in the past few years and to the accumulation of large sets of contradictory data, all of which have been extensively reviewed [15–18].

The lack of critical (bio)chemical insight into the observed biological effects has further clouded this new field of research. For example, there is still not a valid consensus on how the "donors" of H_2S should be handled. Widely used inorganic sources (NaHS and Na_2S) are unstable, prone to further sulfide oxidation and very hygroscopic [19,20]. They also do not qualify to be called donors because they simply create the H_2S/HS^-, H^+ equilibrium when dissolved in water and do not actually release or donate H_2S. However, it is exactly the impurities present in these sources that can cause different biological outcomes [21,22]. For example, it has been proposed that some of the physiological effects of H_2S may be due to its ability to modify cysteine residues and form protein persulfides [23–25]. The direct reaction of H_2S with cysteine residues is not possible without an oxidant being present to either start or catalyze this reaction [26,27]. Polysulfides, which are inevitable contaminants of H_2S solutions [21], could act as oxidants capable of directly oxidizing free cysteine residues in the proteins or as reductants capable of reducing intra- and inter-molecular disulfides. Thus, the purity and stability of H_2S solutions could make a huge difference in the observed channel activation or enzyme inhibition.

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Dihydrogen sulfide can readily diffuse through membranes, but the hydrogen sulfide anion cannot [28,29]. However, in an acid-base equilibrium at physiological pH, it is irrelevant which of the species is the reactive one, H_2S or HS^-. In cultured cell monolayers, the pH of the sulfide solutions will not matter because diffusion into the cells will not be significantly different even at pH 5 [29]. This is best exemplified in superoxide dismutation by SOD encapsulated in micelles, where the lipid barrier does not affect the catalytic rate constant for SOD even at pH 7, when almost all the superoxide molecules are in the anionic form [30]. Independent of H_2S, however, the pH of the medium can affect other cellular functions [31].

The actual physiological concentration of H_2S is still a matter of debate, but low nM concentrations seem most plausible with the possible exception of the aorta, where the concentration is ~20–100-fold higher [32–34]. These low, steady-state levels (despite the fact that the flux of sulfur into H_2S in murine liver is comparable to that of glutathione) suggest that the sulfide clearance rate is high [35–37]. This is not surprising because H_2S is still a toxic molecule with high binding affinity for cytochrome c oxidase [38,39]. This fact is often neglected in experiments where cells and tissues are sometimes exposed to very high (local) concentrations of H_2S.

The striking resemblance of the biological effects triggered by H_2S to those signaled by NO led to the establishment of a new field of study dealing with the cross-talk of these two signaling molecules [18,40–46]. NO inhibits the enzymatic production of H_2S by cystathionine beta synthase [47], where as H_2S can act as a scavenger of peroxynitrite [48]. H_2S can act as a scavenger of peroxynitrite [49], forming sulfinyl nitrite (HS(O)NO), which further decomposes to form NO [50]. H_2S could also react with *S*-nitrosothiols to give the smallest *S*-nitrosothiol, HSNO [51]. The latter can act as a carrier of NO^+ moieties to promote protein-to-protein transnitrosation, or it may further react with H_2S to form nitroxyl (HNO), an NO sibling with distinct signaling properties [52]. Finally, H_2S can affect the nitrite-reductase potential of the cells, either by interfering with the xanthine oxidase system [53] or by reacting with iron-heme centers [54]. However, H_2S can also interfere with some of the methods commonly used to measure NO and its metabolites. For example, it has been demonstrated that depending on the instrumental setup, H_2S disturbs the detection of NO in chemiluminescence assays [55].

Taking all this into account, we designed a study to investigate if and how, when working with H_2S, experimental outcomes depend on its source, dose and the methodology employed. We also provide chemical insights into some of the reactions that had never been studied in detail but were claimed to occur, including direct reactions with cytochrome c, superoxide and nitrite.

2. Material and methods

2.1. Solutions

All solutions were prepared using nanopure water. All the buffer solutions were mixed with Chelex-100 resins, stirred overnight and kept above the resins. In addition, neocuproine (0.1 mM) was used to remove traces of copper ions.

2.2. UV–vis spectrophotometric studies

All spectrophotometric studies were done employing an HP 8452A diode array spectrophotometer connected to a computer equipped with Olis SpectralWorks software. Anaerobic measurements were performed in anaerobic cuvettes.

2.3. Stopped-flow measurements

Kinetic data were obtained by recording time-resolved UV–vis spectra using a modified μSFM-20 Bio-Logic stopped-flow module equipped with a J&M TIDAS high-speed diode array spectrometer with combined deuterium and tungsten lamps (200–1015 nm wavelength range). Solutions were delivered with 10 mL gas-tight Hamilton syringes. The syringes were controlled by separate drives, allowing variation of the ratio of mixing volumes used in the kinetic runs. Data were analyzed using the integrated Bio-Kine software version 4.23 and the Specfit/32™ program. At least five kinetic runs were recorded for all conditions, and the reported rate constants represent the mean values. All kinetic measurements were carried out under pseudo-first-order conditions. The cyt c concentration was kept constant at 10 μM, whereas the H_2S concentration was varied between 100 μM and 2 mM. For anaerobic conditions, the cytochrome c (10 μM), Mn(pyane) (1 mM) and H_2S solutions were prepared in a glove box using argon purged KPi buffer (50 mM, pH 7.4) and gas-tight Hamilton syringes. Spectra were collected every 0.5 ms for different time intervals.

2.4. Analysis of NO and H_2S

The reaction of H_2S and nitrite was monitored by a Free Radical Analyzer (WPI) connected to a Dell computer equipped with DataTrax software for the signal processing. Experiments were performed in a four-channel chamber (WPI) with both electrodes at the same time or each of them separately. The electrodes were immersed in 2 mL of 50 mM KPi buffer, pH 7.4, in the chamber. Depending on the type of measurement, different concentrations of Na_2S solution and/or nitrite were injected.

2.5. GC–MS

GC–MS analyses were performed using a Bruker GC-450 TQ-MS-300. The gas chromatograph was equipped with a Varian VF-5 ms capillary column. Following the previously reported protocol [51], 20 μL of the gas phase from a sample was injected in splitless mode.

2.6. NMR measurements

Spectra of ^{15}N-labeled sodium nitrite (10 mM), before and after addition of sodium sulfide (10 mM), in 300 mM potassium phosphate buffer (pH 7.4) were recorded using a Bruker 400 MHz spectrometer (reference ^{15}N-nitromethane, at 50.67 MHz, 35° pulse width, 5 s relaxation delay) during 24 h [51].

2.7. SDS–PAGE of immunoglobulins

To visualize the reducing properties of differently prepared H_2S solutions, non-reducing SDS–PAGE was employed. Immunoglobulins were dissolved in phosphate buffer, pH 7.4, to a final concentration of 1 μg/mL. The immunoglobulins were obtained from human serum (mostly IgM, IgG and IgA) [56] and incubated with various agents (NaHS, Na_2S, Na_2S + $CuSO_4$ and K_2S_x). Samples were incubated with the desired concentration of Na_2S for 30 min at room temperature and boiled in sample loading buffer without reducing agents (no DTT, no 2-mercaptoethanol) for 7 min. Samples were loaded on 12% polyacrylamide gels and run in SDS-buffer. Fixed proteins were stained with Coomassie staining solution. Experiments were performed in duplicate and documented with a Nikon D800 camera. Images were corrected for brightness, color saturation and contrast with Photoshop (Adobe).