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Review

Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood?

Kenneth R. Olson *

Indiana University School of Medicine-South Bend, 1234 Notre Dame Avenue, South Bend, IN 46617, USA

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ABSTRACT

Hydrogen sulfide (H_2S) is gaining acceptance as a signaling molecule and has been shown to elicit a variety of biological effects at concentrations between 10 and 1000 μmol/l. Dissolved H_2S is a weak acid in equilibrium with HS^- and S^{2-} and under physiological conditions these species, collectively referred to as sulfide, exist in the approximate ratio of 20% H_2S, 80% HS^- and 0% S^{2-}. Numerous analyses over the past 8 years have reported plasma or blood sulfide concentrations also in this range, typically between 30 and 300 μmol/l, thus supporting the biological studies. However, there is some question whether or not these concentrations are physiological. First, many of these values have been obtained from indirect methods using relatively harsh chemical conditions. Second, most studies conducted prior to 2000 failed to find blood sulfide in micromolar concentrations while others showed that radiolabeled ^{35}S-sulfide is rapidly removed from blood and that mammals have a relatively high capacity to metabolize exogenously administered sulfide. Very recent studies using H_2S gas-sensing electrodes to directly measure sulfide in plasma or blood, or HPLC analysis of head-space gas, have also indicated that sulfide does not circulate at micromolar levels and is rapidly consumed by blood or tissues. Third, micromolar concentrations of sulfide in blood or exhaled air should be, but are not, malodorous. Fourth, estimates of dietary sulfur necessary to sustain micromolar levels of plasma sulfide greatly exceed the daily intake. Collectively, these studies imply that many of the biological effects of sulfide are only achieved at supra-physiological concentrations and they question whether circulating sulfide is a physiologically relevant signaling molecule. This review examines the blood/plasma sulfide measurements that have been reported over the past 30 years from the perspective of the analytical methods used and the potential sources of error.

Within the past decade the scientific community has changed its opinion of hydrogen sulfide (H_2S) from noxious and toxic gas to a biologically relevant signaling molecule, i.e., a gasotransmitter [71]. Gasotransmitters must fulfill several criteria including an ability to stimulate appropriate effector systems, synthesis or release of the signal that is initiated by physiological stimuli, and the gasotransmitter must produce biological responses at physiologically relevant concentrations. Considerable evidence has accumulated to support at least the first of these criteria. Sulfide (used in this review to denote the sum of dissolved H_2S and HS^-; see below) has been shown to be stimulatory or inhibitory in neurological, cardiovascular, gastrointestinal, genitourinary and endocrine systems and it may have additional modulatory and/or cytoprotective effects [36,41,45,58,65]). Sulfide has been proposed to be both pro- and anti-inflammatory [39,70] and it has even been reported to induce suspended animation in mice [3]. In the mammalian cardiovascular system sulfide dilates systemic [23] and constricts pulmonary [52] vessels. Sulfide has been shown to have protective effects in the heart and brief exposures to endogenous sulfide provides a preconditioning effect protecting the heart against ischemia reperfusion injury [2,4,16]. In non-mammalian systems it variously constricts, dilates or has multiphasic effects on systemic and respiratory vessels [15]. Nearly all of the effects of exogenous sulfide have been observed at sulfide concentrations in the tens to hundreds of μmol/l.

Sulfide is produced in many, if not all, tissues by two cytosolic pyridoxyl 5′phosphate-dependent enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE; a.k.a. CGL and cystathionase, EC 4.4.1.1; reviewed in [32,41,65]). It has recently been shown that mitochondrial, 3-mercaptopyruvate sulfur transferase (3-MPST; EC 2.8.1.2) is important in sulfide synthesis in the brain [60].

Numerous studies in the past decade have reported sulfide concentrations in cerebral spinal fluid, blood and tissues that are also in the ten to several hundred μmol/l. These values have been used to justify the above mentioned biological studies and satisfy the criteria for sulfide as a gasotransmitter. They also suggest a high level of tonic activity of this system. This exuberance has overlooked a few practical aspects of sulfide chemistry and metabolism. First, there is no obvious odor of H_2S gas in blood or exhaled alveolar air even though micromolar sulfide concentrations in blood should result in H_2S concentrations in exhaled air that can be detected by the human nose [18,75]. In fact, these sulfide concentrations are within the range of those reported for human flatus [37]. Second, Furne et al. [18] predicted that the high level of sulfide production necessary to sustain

* Tel.: +1 574 631 7560; fax: +1 574 6317821.
E-mail address: olson.1@nd.edu.

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micromolar plasma sulfide concentrations would consume cysteine at a rate nearly 100 times greater than the daily requirement of cysteine and methionine combined.

Recent studies employing different analytical techniques have not verified micromolar concentrations of sulfide in either blood [61,75] or tissue [18,26]. This raises the question whether sulfide can exist in the circulation as an endocrine-like signal and, perhaps more importantly, if the sulfide concentrations employed in many biological studies are indeed physiologically relevant. The purpose of this brief review is to briefly evaluate the potential for sulfide to serve as a blood-borne biological signal from the perspective of the molecular forms of sulfide potentially present in blood and the methods utilized to identify these forms.

1. Forms of hydrogen sulfide

1.1. "Free" sulfide

Dissolved H_2S gas is a weak acid and in solution exists in the equilibrium $H_2S \leftrightarrow HS^- \leftrightarrow S^{2-}$. With the pK_{a1} ~7.0 and the pK_{a2}>17 [14,51,54] there is essentially no S^{2-} in biological tissues, nearly equal amounts of H_2S and HS^- within the cell, and approximately a 20% H_2S/80% HS^- ratio in extracellular fluid and plasma at 37 °C and pH 7.4. Because of the temperature sensitivity of pK_{a1}, the H_2S/HS^- ratio remains nearly constant in blood and tissues of ectothermic vertebrates over a wide range of body temperatures, e.g., in trout blood at 10 °C the ratio is 15% H_2S/85% HS^- [14,51]. Often for convenience H_2S, HS^- and S^{2-} are referred to as "H_2S", although it is perhaps more appropriate to refer to these species collectively as either "sulfide" or "total free sulfide".

1.2. "Bound" sulfide

A number of mechanisms have also been described in which sulfide can be reversibly "bound" to other molecules and released at a later date. In this way it may be possible to store or transport sulfide in a less labile, non-gaseous state. There is a broad range of sulfur atoms covalently bound to another sulfur atom to form sulfane sulfur. Sulfane sulfur originates in vertebrates from the anaerobic metabolism of cysteine (Cys) by enzymes that include CBS, CSE and 3-MPST (following deamination of Cys to 3-mercaptopyruvate) and rhodanese (2.8.1.1) and exists in a variety of forms including thiosulfate, persulfides, thiosulfonate, polysulfides, polythionate and elemental sulfur [25,68,69]. A variety of sulfane sulfur compounds are often generically termed acid-labile sulfur, non-acid labile sulfur, bound sulfur, tissue-bound sulfur, sulfide liberated by dithiothreitol, and protein-bound or protein-associated sulfur. These labels originated from the conditions under which the sulfide was released [25,69]. This nomenclature is often confusing; for example, Ubuka [69] and Tanabe [66] make a distinction between acid-labile and sulfane sulfur (the latter liberating sulfide when exposed to reducing agents), whereas Iciek and Włodek [25] include the acid-labile sulfur as sulfane sulfur.

Acid-labile sulfide is derived primarily, but not exclusively from iron-sulfur centers in mitochondrial enzymes. It is released under acidic (pH<5), and therefore generally produced under non-physiological conditions. Typically, acid-labile sulfide is assayed colorimetrically with the methylene blue method or following evolution of H_2S into head-space gas and gas chromatography [69].

Sulfide is released from bound sulfane sulfur by reducing conditions such as excess reduced glutathionine (GSH), Cys [68], dithiothreitol (DTT; [73]) or by a combination of Cys and GSH [26]. In the cell this is favored by alkaline conditions and this has been used as an additional criterion to distinguish this form of sulfur from acid-labile sulfur [26]. Sulfane sulfur is most often quantified by cold cyanolysis and colorimetric measurement of ferric thiocyanate or by direct measurement of evolved H_2S into head-space gas and subsequent gas chromatography [69,76], although this is reported to have limited sensitivity and specificity [25]. Iciek and Włodek [25] also point out that the different methods employed to release sulfide from bound sulfur may release sulfide from different stores and are often not all inclusive.

The relationship between acid-labile sulfide and sulfide derived from bound sulfur has been most extensively characterized by Ishigami et al. [26]. Acid-labile sulfide was not released from homogenates of the brain, heart or liver until pH fell below 5.4 and in all instances the amount of sulfide released was greatest at pH 1.5 (~160 nmol/g protein from the brain and heart and 430 nmol/g protein from the liver). Essentially all acid-labile sulfide originated from the mitochondrial fraction and approximately 30 min were required for the maximal rate of sulfide release by acid. It was also observed that acid-labile sulfide was specifically released from brain homogenates by the detergent sodium dodecylsulfate (SDS) and by guanidine. Sulfide from bound sulfur was released by 5 mmol/l DTT from rat brain and liver, but not the heart. Maximum sulfide release occurred between 1 and 2 h in the liver (nearly 6 μmol/g protein) whereas the rate of sulfide release by DTT in the brain continued to increase over the 5 h experiment, up to nearly 2 μmol/g protein. Release of acid-labile sulfide was not affected by DTT, nor was release of sulfane sulfur affected by HCl. Exogenous sulfide (as Na_2S) was rapidly "absorbed" by the liver and heart (≤10 min) and absorbed by the brain over 30 min. Sulfide was not absorbed by bovine serum albumin, lysozyme, and only slowly by fetal bovine serum. The sulfide absorbed by the brain appeared to be confined to the DTT-labile store and was not mobilized by acid, SDS or guanidine. Sulfane sulfur sulfide was also released from mouse brain homogenates by mixtures of Cys and GSH at physiological concentrations (0.1 and 10, or 0.25 and 10 mmol/l, respectively), whereas graded concentrations of Cys (0.1–0.25 mmol/g protein) or GSH (3–10 mmol/g protein) alone, or in other combinations, were ineffective. Nearly all of the sulfane sulfur was found in the cytosolic fraction of the mouse brain.

1.3. Methods for measuring sulfide in plasma or blood

The majority of measurements of blood sulfide have employed one of two general methods, spectrophotometric measurement of methylene blue formed from the reaction of sulfide with N-dimethyl-*p*-phenylenediamine (NDPA) or sulfide anion (S^{2-}) measurement with an ion-selective electrode (ISE). Both methods almost exclusively use plasma, not whole blood. Two common variations of the methylene blue method are often used. In the original indirect method of Stipanuk and Beck [63], and modified by Geng et al. [19], the sample is placed in a closed container that also contains a filter paper wick. The wick is saturated with an alkali such as zinc acetate but is not in contact with the sample. Acid is added to the sample to drive all of the sulfide into the gas phase where it is absorbed onto the wick. The wick is then removed and mixed with the NDPA. Typically, 30–120 min is allowed for gas evolution and absorption onto the wick. In the second, "direct", method, the NDPA and acid (such as trichloroacetic acid; TCA) are added directly to the plasma to precipitate the plasma proteins and immediately develop color.

Sulfide measurement with the ISE requires that all the sulfide is in the form of S^{2-}. This is accomplished by placing the sample in an antioxidant buffer (AOB) comprised of a strong alkali and an antioxidant (typical working strength: 0.5 mol/l sodium salicylate, 0.12 mol/l, ascorbic acid and 2.2 mol/l NaOH). Sulfide is usually recorded 20–30 min after the plasma is mixed with the AOB (direct method). In the indirect method, the sample is acidified and the evolved H_2S gas is carried in a stream of nitrogen into the AOB.

1.4. Potential problems with the methylene blue and ISE methods

Both the methylene blue and ISE methods employ harsh chemical conditions and there is considerable delay between blood collection and sulfide measurement. It should also be noted that it is

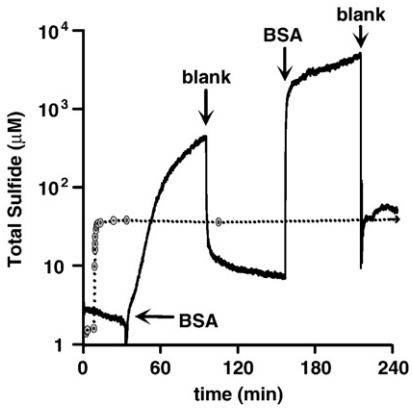

Fig. 1. Continuous recording of sulfide in alkaline antioxidant buffer (AOB) using a sulfide ion-selective electrode (ISE). Sulfide was continuously generated from 5% bovine serum albumin (BSA; solid line) in Kreb's buffer, but not from a 30 μmol/l sulfide standard in BSA-free Kreb's buffer (dotted line). Removing the ISE from the BSA and placing it in protein-free antioxidant buffer (blank) and returning it (second BSA) did not affect the rate of sulfide formation and provided an estimate of the ISE response time. Modified from [75], with permission.

common practice to prepare the sulfide standards in buffer rather than plasma or blood. This avoids potential interfering substances, but it does not accurately reproduce the blood/plasma environment. Other potential problems are more specific to the individual methods. The strong acidic conditions associated with the methylene blue method suggest that if acid-labile sulfide is present in the blood or plasma it would be liberated during the assay along with free sulfide and artificially inflate the sulfide concentration. The alkaline conditions of the AOB have been known to affect ISE measurements in plasma since they were initially employed. Khan et al. [33] observed that directly mixing blood and AOB resulted in protein desulfuration and produced artificially elevated sulfide values. In fact alkaline lability of protein sulfur has been known for some time [5]. We [75] also observed that sulfide was produced directly from bovine serum albumin in this alkaline environment. As shown in Fig. 1, when a sulfide standard prepared in protein-free buffer was placed in the AOB, the ISE recording was stable for 4 h. However, when 5% bovine serum albumin (BSA; ~plasma concentration) was placed in AOB, the total sulfide measured with the ISE rapidly increased for the first 20 min and then continued to slowly increase over the ensuing 3 h. The sulfide concentration in the BSA solution estimated at the 20 min shoulder in Fig. 1 is around 100 μmol/l and this is within the range of sulfide concentrations recorded in plasma samples in many studies over the past 10 years (Table 1). After 3 h of incubation in AOB, the sulfide concentration approached 6–7 mmol/l (Fig. 1). Assuming approximately 35 cysteine molecules per albumin molecule, the 5% BSA solution would contain approximately 27 mmol/l cysteine sulfur. Thus in 3 h this is equivalent to desulfurating nearly one-fourth of the Cys in BSA. While albumin may contain a small quantity of sulfane sulfur, it seems unlikely that this could account for the total sulfide measured with the ISE. These findings suggest that much of the sulfide measured with the ISE is derived from hydroxyl replacement of cysteine sulfur in the albumin. Anecdotally, it was also noticed that when HCl was added to the BSA/AOB several hours later to lower the pH, the strong odor of H_2S was immediately apparent.

1.5. Direct measurement of H_2S gas with the polarographic H_sS sensor

The recent development of a polarographic H_2S sensor has provided an alternative method for sulfide measurement [13,35]. This sensor, patterned after the Clark type oxygen electrode, has an H_2S-permeable membrane, operates at a polarizing voltage well below that of O_2 and appears quite specific for H_2S gas. The polarographic sensor permits the direct measurement of H_2S gas in biological fluids without sample modification and in real-time [13,35,53,75]. Because the polarographic sensor only measures H_2S gas, sulfide (H_2S gas, HS^- and S^{2-}) is estimated indirectly from pH. Sensitivities as low as 15 nmol/l for H_2S (~100 nmol/l sulfide) have been reported [75]. The findings from recent studies using the polarographic sensor have begun to question whether sulfide does indeed exist in the blood and if sulfide (or H_2S gas) is a physiologically relevant "gasotransmitter" in the circulation [75].

2. Evidence against free sulfide in the blood

2.1. Historical perspective

Fig. 2 summarizes the frequency of blood sulfide concentrations reported over the past 29 years. It is evident that while studies prior to 2000 reported undetectable or minimal sulfide concentrations in the blood, since 2000 there has been a tendency for steadily increasing plasma sulfide values. Table 1 provides a chronological list of the sulfide concentrations reported for plasma or blood over this period and the method used. In the 11 studies from 1980 to 2000, 9 reported undetectable levels of sulfide in the blood and the remaining 2 reported 2 and 6 μmol/l. In contrast, 30 of the 39 studies performed in the past 8 years have reported blood or plasma sulfide concentration between 3 and 300 μmol/l, with most in the 30–50 μmol/l range. It is also evident from Table 1 that the high values for plasma sulfide are consistently obtained using either the methylene blue method or the ISE.

As the methylene blue method involves strong acidification, sulfide measurements might be biased by the inclusion of acid-labile sulfide. As shown in Table 1, there were 25 reports of blood or plasma sulfide measurement since 2000 that employed either the direct or indirect methylene blue method. Of these, there were 6 reports of plasma sulfide levels ≥290 μmol/l, 11 studies reported sulfide levels between 20 and 60 μmol/l, and only 4 studies reported sulfide levels ≤ 10 μmol/l. With the exception of studies by Whitfield et al. [75] and Sparatore et al. [61], the range of these measurements was from 8 to 301 μmol/l with an average of 121 μmol/l. In our study [75], the average sulfide concentration measured in plasma from 6 vertebrates using the indirect methylene blue method was 2 μmol/l (ranging from not detected to 4.3 μmol/l) and using HPLC analysis of methylene blue Sparatore et al. [61] reported plasma sulfide <0.55 μmol/l.

There are approximately 15 reports of plasma sulfide concentrations measured using the ISE (Table 1). Of these, only one is in excess of 100, one is less than 30, and the average (excluding the high value) is 48 μmol/l. While there is less variation in these values compared to the methylene blue method, it is possible that the very alkaline antioxidant buffer could release protein-bound cysteine sulfur (see above).

2.2. Radiolabeled ^{35}S-sulfide

In two elegant, but apparently overlooked studies, Curtis et al. [10] and Bartholomew et al. [1] examined the fate of ^{35}S-labeled sulfide in the rat and its consumption by blood and tissues. When injected intravenously, less than 11% of the ^{35}S-sulfide remained in the blood after 5 min. 5 min after addition of ^{35}S-sulfide to whole blood in vitro, over 70% of the injected radionuclide was bound to proteins with approximately half of the ^{35}S bound to protein in the plasma and the remaining half to protein in the red blood cells. When ^{35}S-sulfide was preincubated with whole blood and then perfused through an isolated liver, the ^{35}S-sulfide was rapidly oxidized to ^{35}S-sulfate leaving little free ^{35}S-sulfide. ^{35}S-sulfide oxidation was also organ-specific being extremely rapid in the perfused liver, less so in the kidney, and minimal in the perfused lung. These results provide additional evidence that free sulfide does not circulate in the blood and they also suggest that although sulfide may transiently bind to blood proteins, it can be rapidly unloaded and oxidized by organs such as the liver.

Table 1
Summary of studies reporting plasma or blood sulfide, listed chronologically.

Animal	Method	[Sulfide] (μM)	Change in blood [sulfide] noted	Lower detection limit (μM)	Reference
Steer blood	ISE indirect (METH)	2	N/A		[33]
Wistar rat blood	GC-ECD alk ext dx PFBBr, (METH)	N/D	N/A	0.3	[29]
Rat blood	GC-EDC	N/D			[49]
human serum	HPLC dx MBB, (METH)[a]	N/D	N/A	0.1	[67]
Rabbit blood	GC-ECD alk ext dx PFBBr,	N/D	500–1000 ppm by inhalation (lethal) = 0.012 μmol/g, 200 ppm (non lethal) = N/D	0.3	[30]
Human plasma	HPLC g.d., dx phen, (METH)	N/D	Added 1 and 5 μmoles in vitro, recovered most	0.03	[50]
Human serum	HPLC dx DEAT, (METH)[b]	0.03	N/A		[48]
Human blood	Titrate with I_2 microdist, NaOH trap, (METH)	N/D	Added 5, 10, 15 μg/50 ml, recovered all	0.13	[57]
Human blood[c]	GC-ECD alk ext dx PFBBr,	N/D	Plasma 7 and 30 μmol/l in fatal poisoning	0.3	[31]
SD rat serum[d]	GC (none)	N/D	N/D after gas infusion into cecum	≤1	[38]
Human blood	IC-ECD microdist, (METH)	2–6, 42,47,52[e]	No change when changing protein in diet		[59]
SD rat plasma	ISE	45.6	N/A		[85]
Human blood	GC–MS alk ext dx PFBBr, (METH)	53.5	In vitro spiked baseline 53 μmol/l by additional 12.5, 25, and 37.5 μmol/l and recovered most		[24]
Wistar rat plasma	MB direct (none)	301	Drop to 192 in hypoxia, up to 317 in hypoxia + NaHS		[81]
Wistar rat plasma	?	38.6	Decreased ~50% with L-NAME treatment		[86]
Trout plasma	ISE direct	38	N/A		[14]
Trout plasma	MB direct	40	N/A		[14]
Wistar rat plasma	MB indirect	60	Drop to ~20 with isoproterenol treatment		[19]
SD rat plasma	MB direct	28.9	Increased to 37 after hemorrhage		[46]
SHR rat plasma	MB direct	20	Compare SHR to WKY control (below)		[78]
WKY rat plasma	MB direct	48	See above		[78]
Wistar rat plasma	MB direct	294	Drops to 196 in hypoxia, up to 323 in hypoxia + NaHS, down to 142 in hypoxia + PPG		[84]
Human plasma	ISE direct	34	55% higher in stable COPD patients		[9]
Wistar rat plasma	MB direct	290	Increased to 369 in 45.2 2 °C temperature-induced seizures, decreased to 126 with seizures + HA, increased to 456 with seizures and NaHS		[22]
Human plasma	ISE direct	51.7	Decreased to 26 in heart disease		[27]
Mouse plasma	MB direct	34	Increased to 41 6 h after LPS, and 65 24 h after LPS		[40]
Human plasma	MB direct	43.8	Increased to 150 in humans with septic shock		[40]
SD rat plasma	MB direct	40	Decreased to 38 with streptozocin (stz) treatment and increased to 42 with stz + insulin		[80]
Wistar rat plasma	ISE indirect[f]	47	Decreases to ~37 with colorectal distension (i.e. pain), 29 with i.p. NaHS, 18 with NaHS + L-NAME		[12]
Human child plasma	ISE direct	65	Decreased to 52 in children with essential hypertension		[11]
Wistar rat plasma	MB direct	298	Decreases to 195 with hypoxia, 271 with i.p. NaHS + hypoxia		[28]
Rat	MB direct	274–295			[21]
SD rat plasma	ISE	29	"H2S solution" increased to 40, hyperhomocysteinemia increased to 50		[6]
Human child plasma	ISE direct	52	N/A		[7]
SD rat blood	PH_2SS	≤5	N/A		[34]
Sw. alb. mouse plasma	MB direct	8	Increases to 20 with cecal ligation/puncture		[82]
Wistar rat plasma	MB direct	38	Increased after myocardial infarction as follows (all i.p.): vehicle 57, PPG 39, NaHS 92		[87]
Trout whole blood	PH_2SS	N/D	Drops to 0.3 after 10 10 μmol/l sulfide spike	0.015	[75]
Trout plasma	MB direct	3			[75]
Mouse whole blood	PH_2SS	N/D	Drops to 0.5 after 10 10 μmol/l sulfide spike	0.015	[75]
LW rat whole blood	PH_2SS	N/D	Drops to 0.4 after 10 10 μmol/l sulfide spike	0.015	[75]
LW rat plasma	MB direct	N/D			[75]
HSD rat whole blood	PH_2SS	N/D		0.015	[75]
HSD rat plasma	MB direct	4			[75]
Pig whole blood	PH_2SS	N/D	Drops to 0.3 after 10 10 μmol/l sulfide spike	0.015	[75]
Pig plasma	MB direct	2			[75]
Cow whole blood	PH_2SS	N/D	Drops to 0.4 after 10 10 μmol/l sulfide spike	0.015	[75]

(continued on next page)

Table 1 (*continued*)

Animal	Method	[Sulfide] (μM)	Change in blood [sulfide] noted	Lower detection limit (μM)	Reference
Cow plasma	MB direct	2			[75]
Trout blood in vivo	PH_2SS	N/D	Drops to 0.2 after 30 30 μmol/l injection	0.015	[75]
Trout blood in vivo	PH_2SS	N/D	No change after hypoxia	0.015	[53]
Trout blood	ISE direct	391	Hypoxia inc to 1 mmol/l		[53]
Mouse serum CSE+/+	ISE direct	41			[79]
Mouse serum CSE−/+	ISE direct	33			[79]
Mouse serum CSE−/−	ISE direct	17			[79]
Rat plasma	MB indirect	33, 47			[42]
Rat plasma	ISE direct	31	Ovalbumin inc to 72, ova + i.p. NaHS 40		[8]
Rat plasma	MB/HPLC	0.55	1.42 with H_2S-releasing asprin		[62]
Human plasma	?	45	Alzheimer's = 34, vascular dementia = 36, cerebrovascular disease = 37		[44]
Mouse plasma	MB direct	10	+PPG = 13, cecal puncture puncture = 20, puncture + PPG = 13, sham = 14		[83]
Rat plasma	ISE direct	37	Oeloc acid = 20, oelic acid + NaHS = 27		[43]
Rat plasma	MB direct	300	Hypoxia = 187, hypoxia + NaHS = 317		[74]
Zucker rat plasma	ISE direct	48 (lean)	Diabetic = 68, fatty = 48, after PPG all ~20		[77]
Mouse	ISE direct	58	ApoE−/−45, AopE−/−+NaHS = 52, ApoE−/−+PPG = 36		[72]
Trout plasma	ISE direct	90	Hypoxia = 420		[55]
Rat plasma	MB/HPLC	<0.55			[61]

Summary does not include studies that involve cases of lethal sulfide poisoning or in which the reductant dithiothreitol was added to the sample prior to analysis. Concentrations are control values unless noted. Question mark in the "Method" column, method not clear or unavailable.

Abbreviations: alk ext, alkaline extraction; dx DEAT, derivatization of 2-amino-5-N,N-diethylamino toluene to thionine; dx MBB, derivatization of monobromobimane to fluorescent sulfide derivative; dx PFBBr, derivatization of pentafluorobenzyl bromide to bis(pentafluorobenzyl)sulfide; dx phen, derivatization of *p*-phenylenediamine to thionine; METH., paper is describing a new method for measuring sulfide in blood or plasma; ECD, electrochemical detector; GC-ECD, gas chromatography-electron capture detector; GC–MS, gas chromatography–mass spectrometry; g.d., gas dialysis; HA, hydroxyl amine, cystathionine β-sythase inhibitor; HPLC, high performance liquid chromatography; IC, ion chromatography; ISEdirect, sulfide ion-selective electrode, sample is added directly to antioxidant buffer; ISEindirect, sulfide ion-selective electrode, sample is acidified and H_2S carried to antioxidant buffer in a stream of N_2; LPS, lipopolysaccharide-induced inflammation; MBdirect, methylene blue, sample is added directly to assay reagents; MBindirect, methylene blue, H_2S is liberated from sample by acid, trapped as ZnS on filter paper and added to assay reagents; MB/HPLC, methylene blue, high performance liquid chromatography; microdist, microdistillation; N/D, not detected; PH_2SS, polarographic hydrogen sulfide sensor; PPG, propargylglycine, cystathionine γ-lyase inhibitor; SD, Sprague–Dawley; SHR, spontaneously hypertensive rat; Sw. alb., Swiss albino; WKY, Wistar–Kyoto.

Superscripts:

[a] Plasma was ultrafiltered 15 min before assay.

[b] Let blood stand for 2 h before taking serum.

[c] Blood came from patients with non-fatal sulfide poisoning.

[d] Cecum was inflated with H_2S gas during blood draw.

[e] Six values of 2–6 μM were listed in a table while 42, 47, and 52 μM were reported in the text. The reason for the difference was not clear.

[f] Add NaOH to sample prior to acidification, which could lead to desulfuration of protein as in direct ISE method. Modified from [75], with permission.

2.3. Measurements with the polarographic H_2S sensor

In a recent study, we [75] used the polarographic H_2S sensor to measure sulfide in plasma and blood from a variety of vertebrates. This study failed to find evidence that H_2S gas exists in the blood or plasma at the level of sensitivity of the sensor (15 nmol/l) and it appeared that exogenous sulfide was in fact consumed by blood in vitro (Fig. 3). In addition, this [75] and another study [53] fitted rainbow trout with an extracorporeal loop and pumped blood from either the dorsal (post-gill) or ventral (pre-gill) aorta of unanesthetized fish across a thermo-jacketed polarographic sensor and returned it into the caudal vein. Both studies failed to detect sulfide in the blood. Furthermore, injection of exogenous sulfide into the fish produced a transient spike in blood sulfide but this returned to baseline within 30 min (Fig. 4; [75]).

It is interesting to note that in a study of a novel water-soluble H_2S-releasing molecule, GYY4137, Li et al. [42] used the polarographic H_2S sensor to measure release of sulfide from GYY4137 when the GYY4137 was dissolved in phosphate buffer, whereas, they used the methylene blue/zinc acetate trapping method to measure sulfide in plasma. It is not clear why these two different methods were used. Li et al. [42] also observed that an intravenous injection of 20 μmol NaHS per kilogram did not significantly increase plasma sulfide 30 or 180 min after injection (measured with the methylene blue method). However, six other studies that employed the methylene blue method (Table 1) reported that intravascular or intraperitoneal injection of sulfide increased plasma sulfide (all but one of these reported control plasma sulfide levels was in excess of 200 μmol/l).

2.4. Anecdotal evidence

A number of other early reports anecdotally and experimentally support rapid consumption of sulfide by blood in vivo and in vitro and they suggest that free sulfide levels are indeed very low. In pioneering studies, Haggard [20] showed that if 10 ml of 77 mmol/l Na_2S was rapidly injected into a dog it was invariably lethal whereas five times that dose could be injected if given over a 20 min period and the animal was "apparently none the worse." Haggard concluded that sulfide was rapidly detoxified, presumably through metabolism. Prior et al. [56] observed that LC_{50} (concentration at which 50% of the subjects died) for H_2S inhaled by rats was 335 ppm over 6 h of exposure while the LC_{10} over the same time was 299 ppm. Assuming that H_2S readily equilibrates across the alveolar membranes, this would produce sulfide values in the plasma of 157 μM and 143 μM, respectively (calculations in [75] "supplementary material"). In fact, Prior et al. [56] suggested that the steepness of the dose–response curve was due to an overload of the sulfide detoxification system,

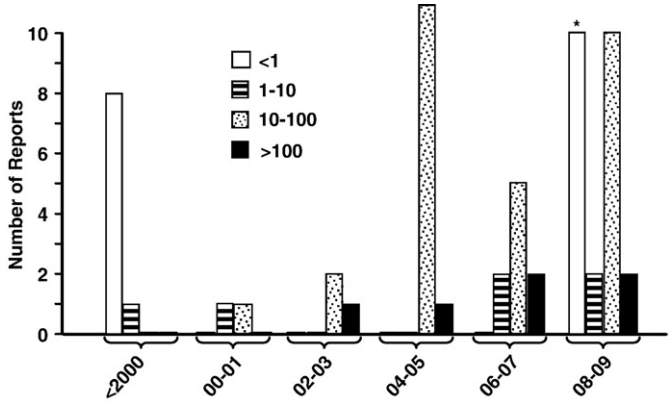

Fig. 2. Frequency distribution of recorded sulfide concentrations in blood from different vertebrates over the past 30 years. Bars indicate the range of sulfide concentration in μmol/l. *, 8 of the 9 values less than 1 μmol/l reported in 2008–2009 were obtained using the polarographic H_2S sensor. Note the trend for increasing plasma sulfide values over the past 10 years.

implying that the majority of the lower dose is being continuously detoxified. Given this capacity to metabolize sulfide, it is difficult to imagine that there would be any opportunity for sulfide to accumulate under resting conditions. It is also clear that H_2S can leave the blood across the alveolar membrane, as intravenously injected NaHS was partially recoverable as H_2S in exhaled air [17]. If 10 μM sulfide in plasma was in equilibrium with alveolar gas, H_2S should be readily detectable by smell in exhaled air where its concentration would be 22 ppm. Clearly it is not noticeable. Moreover, the reported levels of

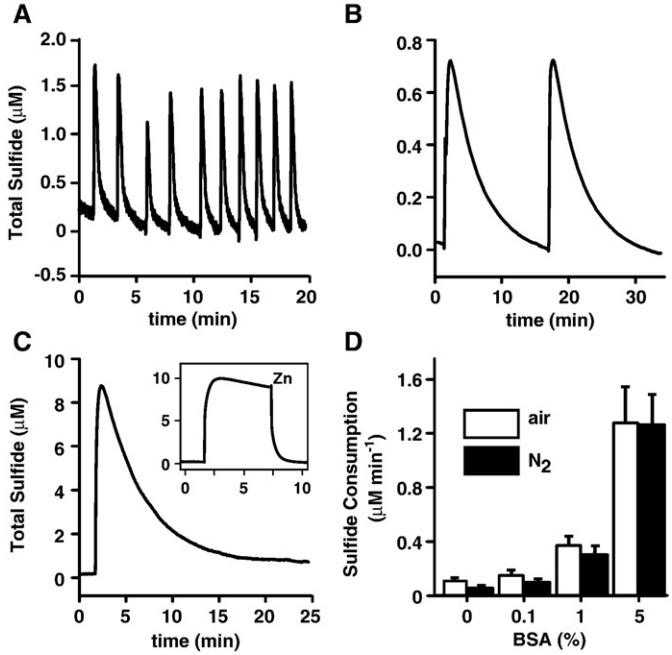

Fig. 3. Polarographic measurement of exogenous sulfide (Na_2S) consumption by whole blood from trout (A), Harlen–Sprague–Dawley rat (B) or 5% bovine serum albumin (BSA) in Kreb's buffer (C). Each spike represents enough Na_2S to raise total sulfide 10 μmol/l, i.e., trout blood will increase to100 μmol/l, rat blood to 20 μmol/l and BSA to 10 μmol/l if there was no sulfide consumption. Inset in (C); sulfide added to Kreb's buffer without protein remains in the solution but is rapidly removed by the addition of excess Zinc acetate (Zn; final concentration 450 μmol/l). (D); sulfide consumption in BSA increases with increasing protein concentration and is independent of the presence of oxygen (open bars equilibrated with 95% air/5% CO_2, closed bars equilibrated with 95% N_2/5% CO_2; all $n=4$). Modified from [75], with permission.

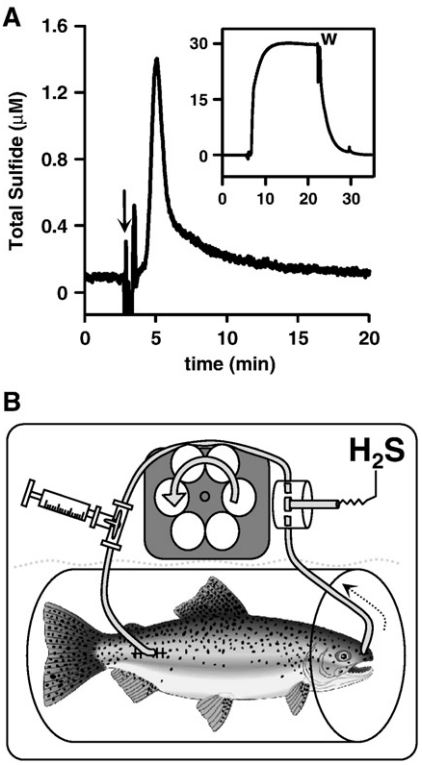

Fig. 4. Real-time measurement of plasma sulfide in an unanesthetized rainbow trout fitted with an extracorporeal loop (A). Blood was pumped from a cannula inserted into the dorsal aorta across a thermo-jacketed polarographic H_2S sensor and returned to the fish via the caudal vein (shown schematically in B). Sulfide estimated from H_2S gas measurement and blood pH (7.8) of resting trout was ~0.1 μmol/l. At 3 min (arrow) exogenous sulfide (as Na_2S), theoretically sufficient to raise plasma sulfide to 30 μmol/l when fully mixed in the plasma, was injected as a single bolus into the caudal vein. Following injection, plasma sulfide increased to ~1.5 μmol/l and rapidly returned to baseline. These results show that circulating sulfide in trout blood is essentially nil and when exogenous sulfide is injected into the vasculature it does not remain in the circulation longer than a few minutes. Inset: injection of the same amount of sulfide into a recirculated volume of physiological buffer equivalent to the trout's plasma volume and pH. Sulfide concentration remains stable for 15 min until washed away (W). (A), modified from [75], with permission.

H_2S in human exhaled [47] and end-expiratory [64] air are only ~50 ppb, more than 400-fold less than predicted.

3. Evidence against labile sulfide transport in blood

We have used a variety of procedures to determine if sulfide consumed by blood is incorporated into a labile pool and/or if this is a methodological artifact. Using the direct methylene blue method, we measured sulfide in whole blood and plasma spiked with Na_2S [75]. As shown in Table 2, if plasma is spiked with sulfide (as Na_2S) after acidification and centrifugation, essentially all of the sulfide in the spike is recovered. However, if the sulfide is added to whole blood

Table 2
Acid-labile sulfide and sulfide recovery from fresh plasma and Na_2S-spiked plasma or spiked whole blood assayed with the direct methylene blue method.

	Plasma	Spike	Spiked plasma	Spiked blood	*N*
Lamprey	2.6 ± 0.6	10	10.2 ± 0.6	1.2 ± 1.0	4
Trout	1.6 ± 0.5	10	10.8 ± 0.5	2.0 ± 0.1	8
HSD rat	4.3 ± 0.5	10	10.4 ± 0.7	NM	6
Pig	2.3 ± 0.2	10	8.6 ± 0.2	1.5 ± 0.1	4
Cow	2.1 ± 0.4	10	9.4 ± 0.8	1.1 ± 0.1	4

Mean ± SE, N = number of animals; NM, not measured. Na_2S was added to plasma after centrifugation and acidification and to whole blood prior to centrifugation and acidification. In both instances the amount of Na_2S should theoretically increase the sulfide concentration by 10 μmol/l. Adapted from [75], with permission.

prior to the acidification step, none of the added sulfide is recovered. These studies suggest that sulfide is rapidly consumed by whole blood (which confirms the measurements with the polarographic H_2S sensor, see above) and that the added sulfide does not become part of an acid-labile pool. Similar findings have recently been reported by Sparatore et al. [61].

To determine if exogenous sulfide could be absorbed by blood and then released by the antioxidant buffer, we incubated trout blood for 30 min after being spiked with an amount of NaHS calculated to produce a theoretical increase in sulfide concentration of 50, 100 and 250 μmol/l [53]. Sulfide concentrations in the spiked samples were 0, 5 and 88 μmol/l suggesting that the exogenous sulfide was not incorporated into an alkaline-labile sulfur pool and that some of the exogenous sulfide was actually removed from the free sulfide pool. Because these samples were not aerated it is unlikely that the sulfide was lost as H_2S gas and more likely that it was oxidized.

4. Is there a "history" of sulfide in the blood?

In spite of the experimental and anecdotal evidence that H_2S gas or sulfide does not circulate in vertebrate blood, the numerous reports that a variety of experimental protocols appear to significantly affect plasma or blood sulfide concentrations (Table 1) is particularly disconcerting. Does this suggest that all of these results are experimental artifact? Or, do these results collectively suggest that sulfide is carried in some form other than either acid-labile or sulfane sulfur, that this unknown form can be detected by both methylene blue and ISC methods, and that this provides a history of past sulfide production? Obviously, resolution of this question is of paramount importance in evaluating the biological and potentially clinical significance of sulfide.

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