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The Role of Hydrogen Sulfide in Evolution and the Evolution of Hydrogen Sulfide in Metabolism and Signaling

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The chemical versatility of sulfur and its abundance in the prebiotic Earth as reduced sulfide (H_2S) implicate this molecule in the origin of life 3.8 billion years ago and also as a major source of energy in the first seven-eighths of evolution. The tremendous increase in ambient oxygen ~600 million years ago brought an end to H_2S as an energy source, and H_2S-dependent animals either became extinct, retreated to isolated sulfide niches, or adapted. The first 3 billion years of molecular tinkering were not lost, however, and much of this biochemical armamentarium easily adapted to an oxic environment where it contributes to metabolism and signaling even in humans. This review examines the role of H_2S in evolution and the evolution of H_2S metabolism and signaling.

The simplest definition of life is the ability to utilize and control energy. Today, nearly all of life's energy is derived from the sun. Plants oxidize water to oxygen and reduce inorganic carbon, whereas animals derive energy by reversing this process. Photosynthesis was not an innate property when life originated, and a number of scenarios have been proposed to provide energy and/or energized organic molecules. Hydrogen sulfide (H_2S) is mentioned in most scenarios, but generally as a minor contributor. In this review, we will present arguments suggesting that H_2S had a far greater role in the origin of life and primordial metabolism than previously thought. Remnants of these activities persist in modern animals, not as a primary energy source, but as an important regulator or modulator of metabolism and signaling.

Sulfur and Sulfide Chemistry

Sulfur is the 10th most common element in the universe, the 15th most common in the Earth's crust, and the 7th most common element in animals (53). This biological concentration is indicative of sulfur's considerable utility and versatility in living systems. Sulfur has eight formal oxidation states, −2 to +6, with even integers being the most stable. H_2S (−2), the most reduced, is a weak acid; $H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + H^+$, where pKa_1 is 6.9 and pKa_2 is between 12 and 17 (119). At pH 7.0, dissolved $H_2S \approx HS^-$, whereas S^{2-} is often considered to be essentially negligible, the latter a mistake that ignores the fact that, in an equilibrium, S^{2-} can theoretically be generated until all sulfide (H_2S, HS^-, and S^{2-}) is consumed (75). In cells, the HS^--to-H_2S ratio can change from 12.6 in the mitochondrial matrix (pH 8.0) to 0.006 in acidic lysosomes (pH 4.7). Dissolved H_2S is lipophilic and readily diffuses through membranes (88), essentially creating pH-dependent equilibria on both sides of these barriers; however, ionized species are more chemically reactive. The temperature dependency of the pKa_1 can be described by the equation $pKa = 3.122 + 1{,}132/T$, where T = degrees Kelvin (119). When life began, it is likely that the percent H_2S, HS^-, and S^{2-} in the deep open ocean (~2°C, pH 6.5) would have been 66, 33, ~0%; compared with 56, 43, ~0% in effluent from hot (400°C) acidic (pH 4.5) thermal vents (black smokers) or 4, 94, 1% in cooler (70°C) alkaline (pH 9.5) white smoker thermal vents. Dissolved H_2S is also volatile, reflected by its 5-min half-time in open tissue culture wells, 3 min in aerated myographs, and <1 min in Langendorff perfused heart preparations (24). Nevertheless, its downstream biological effects can persist for hours. Perhaps the greatest single obstacle in the field of H_2S biology is the accurate measurement of intracellular H_2S (77, 121).

A one-electron oxidation of two sulfides or a two-electron oxidation of one of the two sulfides forms the simple persulfide, H_2S_2. Additional oxidative steps form progressively longer polysulfide chains, up to S_8, at which point the sulfur chain is presumed to cyclize and become insoluble (169), although this is not always the case (see below). Polysulfides can act as either a reductant or an oxidant, a point considered in greater detail later. pKa_1 and pKa_2 for H_2S_n rapidly decreases as n increases (60), potentially increasing reactivity.

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A Brief History of the Earth

The earth was formed ~4.6 billion years ago (bya), and it is defined by four eons. The Hadean Eon, named after Hades, was inhospitable, excessively hot, and anoxic. If life began here, it would have been destroyed by extraterrestrial impacts of unimaginable magnitude and frequency, but these would have also brought life's essentials, water, an atmosphere, and organic molecules (156). Life began early in the Archean eon (3.8 bya; FIGURE 1) in a warm and ferrungous (anoxic and Fe^{2+} dominated) ocean (138, 149). The Proterozoic eon began 2.5 bya. Oxygen appeared in the atmosphere ~2.3 bya, the "great oxidation event" (GOE) in which atmospheric oxygen may have increased several times to ~2% while the oceans remained essentially anoxic. Evolution of modern-day plants, some 600 million years ago (mya), ushered in the Phanerozoic eon and the tremendous biomass that could only be supported by solar energy and an abundance of atmospheric oxygen.

Origin of Life

Theories of life's origin follow two main themes: Where did the first organic molecules come from and how was energy harnessed to drive metabolism? Stanley Miller was the first to suggest that lightning could have provided the energy to create the "primordial soup" (98). Other sources of organic molecules include high-energy nuclear reactions in far-off stars then delivered in comets, meteors, or cosmic dust (127, 130, 131), and photocatalyzed reactions in the atmosphere (147). H_2S is present in all of these possibilities, even in recently discovered samples from Miller's original experiments (126, 127). While all of these theories provide organic precursors, they cannot consistently deliver useful energy, and dispersion of the initial products in the ocean or atmosphere limits the probability of coupled, sequential chemical reactions. Thus recent attention has turned to hydrothermal vents. In fact, the prebiotic earth has been likened by some to a prototypical cell where energy in the form of reducing equivalents traverses these vents as chemiosmotic gradients do across a cell membrane (89, 136).

Hydrothermal vents are created along the separation lines of tectonic plates, e.g., the mid-ocean ridge-spreading centers. There are two general types, black and white smokers, so named for the color of the vent effluent. Black smokers are close to the spreading centers where magma heats seawater that has seeped into the crust and they emit hot (300-400°C), acidic (pH 2–3) seawater rich in CO_2 (4–215 mm/kg), H_2S (3–110 mmol/kg), dissolved H_2 (0.1–50 mmol/kg), and reduced transition metals, especially iron (Fe^{2+}). Iron and sulfide react in the vent fluid to form FeS, which is then precipitated when it contacts oxygenated seawater, thereby forming the characteristic black particulate plume (64, 82, 144). The combination of high pressure and heat can drive reactions not kinetically possible under other conditions, and when both temperature and pressure decrease as these fluids rise from near the magma toward the seafloor, the stability of more thermally labile products is favored. Heat deep within the smokers keeps metal sulfides in solution, and acidity favors their dissociation and elevates H_2S concentrations (51). In the prebiotic earth, it is quite likely that H_2S and reduced metal sulfides remained in solution in anoxic seawater for prolonged periods and could have spread considerable distances (35). Present-day vents contain the densest biomass on earth, evidence of their abundant energy and the ability of living organisms to use that energy.

The recently discovered white smokers are typically found lateral to the mid-ocean Ridge (off-axis vents) and are alkaline (pH 9–11) and cooler (70–90°C), as magmatic heating is considerably reduced (82, 150). They have high concentrations of H_2 (<1–12 mM) and CH_4 (1–2 mM), but little CO_2 or H_2S. White smokers sit on or near the magnesium- and iron-rich mineral olivine, which, when in contact with seawater, creates an exothermic reaction ultimately generating H_2 through a process known as serpentinization (145). The heat generated by this process also drives a hydrothermal circulation (145). Much recent work has focused on this process as providing the energy and the chemistry for the origin of life in the form of reducing equivalents (H_2) that can then form methane from CO_2 and by creating a chemio-osmotic gradient between alkaline the vent fluid and circumneutral (pH 6.5) seawater (10, 23, 48, 82, 83, 85–87, 89, 113–115, 142, 143, 145, 146, 150, 157, 173, 180). Paradoxically, white smokers support relatively little biomass or diversity (145).

Some vents are more unique and provide evidence for H_2S in life's origins. These vents are found on or near tremendous deposits of metal sulfides, often called sulfide lenses (152, 153). They are relatively hot (200–370°C) because they are heated by both magmatic flow and serpentinization, acidic (pH 3–4), and with high concentrations of H_2S (0.5–2 mM), H_2 (10–25 mM), and CH_4 (0.5–2.5 mM). It is our opinion that these events offer the greatest opportunity for life due to the versatility of sulfide and the many energetic transformations that can occur.

The Multifunctional Role of H_2S at Life's Origin

H_2S was arguably the most versatile molecule when life began because it could serve as an important organic product, reactant, catalyst (proto-enzyme), barrier (proto-membrane), and sustainable source of energy. In the "iron-sulfur world" (172), oxidation of HS^- by FeS, both products of hydrothermal vents, produces a variety of organic molecules (reviewed in Ref. 16) as well as reducing N_2 or nitrate to ammonia and generating amines (9, 27, 63, 116, 162). Sulfide reacts with Fe^{2+} and other transition metal ions, and many of these can serve as unique and gateway catalysts (22, 43, 104, 112, 116, 137). For example, sphalerite (ZnS) is a highly specific catalyst for activation of single carbon-hydrogen bonds (155). Sulfide and iron combinations form minerals such as pyrite (FeS_2), greigite Fe_3S_4 (138, 140), and iron sulfur clusters such as Fe_2S_2 and Fe_4S_4, all of which cannot only act as catalysts but potentially act as physical barriers forming prototypical membranes (89, 92, 93). Iron sulfur clusters are also found in a variety of enzymes and act as chemical "wires" to conduct electrons; 12 are found in mitochondria. Transition metals also react with sulfur to form metal polysulfides, which increases sulfur's reactivity and versatility.

A number of factors support H_2S over H_2 as the primordial energy source. First, there is typically more H_2S than H_2 exhausted from vents. Second, transition metal sulfides (e.g., FeS) can potentially release more H_2S per volume from sulfide lenses (55,000 mol/m^3) than H_2 can be generated from olivine (500 mol/m^3 olivine; Ref. 82). Third, oxidation of H_2S produces more energy than H_2 oxidation

$$H_2S + 4H_2O \rightarrow H_2SO_4 + 4H_2 : -662.7 \text{kJ/mol} \quad (1)$$

vs.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O : +343.8 \text{ kJ/mol} \quad (2)$$

or

$$H_2S + 2H_2O + CO_2 \rightarrow H_2SO_4 + CH_4 : -318.9 \text{ kJ/mol} \quad (3)$$

Fourth, H_2S oxidation generates additional equivalents of H_2 (*Eq. 1*). And fifth, complete oxidation of H_2S to H_2SO_4 releases eight electrons, enough to completely reduce carbon to methane compared with two electrons released by H_2 oxidation.

H_2S and Photosynthesis

The ability to extract energy from a photon and use it to form or break chemical bonds freed organisms from their chemolithotrophic existence and their dependency on reducing equivalents supplied from within the Earth to drive cellular redox chemistry. This likely occurred relatively soon after the origin of life ~3–3.5 bya (49, 149, 156). The initial type-I photosynthetic pathways were sequential two-electron transfer processes mediated by soluble cytochromes and were anoxygenic. Their light-gathering antennae absorbed longer wavelength light and, because water is a weak electron donor, reduced compounds, such as H_2S, H_2, Fe^{2+}, organic carbon, and nitrate, have been suggested as possible electron sources (139). H_2S would not only be a likely candidate because of its abundance, but the molecular similarity to water would be a convenient "lead-in" to more sophisticated high-energy type-II photosynthesis that followed. Relics of H_2S-mediated photosynthesis are present in modern-day anerobic photosynthetic purple and green sulfur bacteria as

$$CO_2 + H_2S + h\nu \rightarrow (CH_2O)_n + H_2O + S_{(n)} \quad (4)$$

where $S_{(n)}$ denotes polysulfides or elemental sulfur that is formed and packed into globules that are either excreted or retained within the cell. The latter may still be important as a means of sulfide storage, trafficking, and signaling as discussed in the last section. Perhaps bespeaking to their primal origins, some extant green anoxygenic photosynthetic bacteria have light-gathering antennae, chlorosomes, tuned to the low-energy infrared radiation emitted from hydrothermal vents (7). As in mitochondria, Fe_2S_2 clusters also assist in electron transfer in chloroplasts (123).

Oxygenic photosynthesis, a four electron oxidation of two water molecules, first appeared in cyanobacteria probably several hundred million years after anoxygenic photosynthesis. This "great oxidation event" (GOE) may have periodically increased atmospheric oxygen to <2% (P_{O_2} of <15 Torr) of present atmospheric levels (pal) ~2.3 bya (19, 28, 50, 149). However, the oceans remained largely anoxic, and recent studies suggest that atmospheric oxygen levels were considerably lower than previously suggested, at most 0.1% of pal (P_{O_2} of <2 Torr) even from 1.8 to 0.8 bya (132). Because the light-gathering antennae of primitive anoxygenic chlorophyll (bacteriochlorophyll) could not collect sufficient energy to oxidize water, it has been proposed that other intermediates such as hydroxyl amine, hydrogen peroxide, hydrazine, nitric oxide, nitrite, or HCO_3 were the "transitional" electron donors leading up to oxygenic processes (49). Raymond and Blankenship (139) suggest that hydrogen peroxide was the most likely intermediate and propose that binuclear manganese catalase ultimately became the tetranuclear manganese on the oxygen evolving complex (OEC) of chlorophyll.

We propose that H_2S or hydrogen persulfide (H_2S_2) would be better "transitional" electron donors than peroxide. The oxidation potential for $H_2S \rightarrow S^0 + 2H^+ + 2e^-$ is -0.14 E^0(V), far less than that of water to peroxide; $2H_2O \rightarrow H_2O_2 + 2H^+ + 2e^-$ [-1.78 E^0(V)] or peroxide to oxygen $H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$ [-0.68 E^0(V)]. There was also considerably more H_2S in the environment than H_2O_2. Using H_2S would also provide a logical transition where H_2S_2 derived from two-electron oxidation of H_2S in anoxygenic photosynthesis could be utilized in a second reaction with H_2S, e.g.

$$H_2S + h\nu \rightarrow 2H^+ + 2e^- + S^0, \text{ then; } S^0 + H_2S \rightarrow H_2S_2 \quad (5)$$

forming progressively longer chain polysulfides. In addition, H_2S could easily have been the antecedent four-electron donor paving the way for its cogener, water

$$2H_2S + h\nu \rightarrow 4H^+ + 4e^- + S_2 \quad (6)$$

Sulfide and the Origin of Mitochondria

The slight increase in atmospheric oxygen during the GOE oxidized terrestrial sulfur to sulfate, which was then washed to the sea. Here, the omnipresent Fe^{2+}, along with the appearance of a few sulfate-reducing organisms (65), reduced sulfate to H_2S, and large areas of ocean became euxinic (anoxic and sulfidic). Eukaryotes first appeared in this environment. The following paragraphs describe the evolution of organisms and metabolic mechanisms that oxidize sulfide; organisms that reduce sulfite and sulfate back to sulfide are considered elsewhere (5, 6).

Eukaryotes require mitochondria to transform oxygen reduction into useful energy. It is most often accepted that mitochondria are derived from a single endosymbionic event ~1.5 bya in which their precursor, an α-proteobacteria akin to *Rickettsia*, was engulfed by a host Archea (21, 28, 81, 165, 178). A novel monophyletic archael phylum "Lokiarchaeota" with genes coding numerous eukaryotic signature proteins is a likely ancestral host (158). Not surprisingly, Lokiarchaeota were found in sediment near the black smoker hydrothermal vent, Loki's Castle (158). A number of advantages have been attributed to such a union. For instance, the "Ox-Tox" model suggests this union prevents oxygen toxicity (72), although an intracellular organelle is not ideally suited to protect the cytosol from extracellular insult. The "hydrogen" hypothesis suggests this as a mechanism of hydrogen transfer (84), although loss of hydrogen from the atmosphere could be problematic. On the other hand, "sulfide syntrophy" (151) suggests a mechanism of sulfur cycling. This is intriguing since it incorporates features of a sulfide-reducing host with the sulfide-oxidizing endosymbiont, an advantageous union in the euxinic ocean where sulfide could provide energy. Sulfur syntrophy is also consistent with sulfur cycling in modern-day eukaryotes (see below), and it reflects the primordial lineage of sulfide-metabolizing enzymes, including some organisms with anaerobic mitochondria (91).

The first three steps in H_2S metabolism in humans and some bacteria are identical, suggesting a long phylogenic relationship (90). Indeed, the enzyme sulfur quinone oxidoreductase (SQR), the first step in H_2S metabolism (see below), not only appears to have been present in the original mitochondrial endosymbiont (167), it is physically embedded in the eukaryotic electron transport chain of extant animals (47). Because many elements of the mammalian electron transport chain as well as SQR predate the emergence of cyanobacteria, and therefore predate oxigenic photosynthesis (12, 13, 39), it seems reasonable to conclude that these systems initially served another energetic pathway, and H_2S oxidation would be the most logical candidate.

The Advent of Environmental Oxygen, Demise of Free Sulfide, and Origin of Modern-Day Animals

Subsequent endosymbiotic events in which eukaryotic cells incorporated cyanobacteria gave rise to modern plants at the beginning of the Phanerozoic (FIGURE 1), ~800 mya (4, 34, 50, 61, 62, 71, 79, 138, 149, 179). The combined activity of cyanobacteria and plants tremendously increased oxygen production, but the oxygen was quickly "mopped up" by the vast amounts of reduced iron and sulfide. This probably took another several hundred million years, but, when finished, the oceans were oxidized, atmospheric oxygen rose to present-day values, and sulfide was effectively eliminated as an energy source. It is generally thought that the rise in oxygen posed a new threat to life, i.e., organisms either developed antioxidant mechanisms to deal with oxygen's toxic effects, retreated to anoxic environments, or became extinct. However, we propose an alternative explanation. Because antioxidant mechanisms were already in place to deal with reactive sulfide species (RSS), they needed to be only slightly tuned to deal with reactive oxygen species (ROS). This allowed animals access to the practically unlimited supply of reduced carbon compounds now provided by plants and to the most potent and abundant electron acceptor, oxygen. The result was a massive explosion in Earth's biomass and complexity.

Sulfide Metabolism in Modern-Day Metazoans

For all practical purposes, the rise in oxygen 600 mya divided eukaryotes into two groups, phototrophs and chemotrophs, the former producing oxygen and reducing inorganic compounds, mainly those of carbon, and the latter, basically consumers, completely dependent on the former's activities. Assimilation and reduction of oxidized sulfur (mainly sulfate and sulfite) by micro-organisms and plants can be found in recent reviews (31, 49, 141) and will not be considered here. Metazoans in general, and vertebrates in particular, which will be considered in detail, typically cannot reduce sulfur compounds more oxidized than S(+2). Thus animals must rely on plants and prokaryotes for these compounds, nearly all of which are incorporated as completely reduced S(−2) sulfur amino acids (S-AA), methionine (the only essential S-AA), and cysteine. For instance, most of the human sulfur intake in Western societies is used for synthesis. The average intake of S-AA is 26 mmol/day, and S-AA from protein turnover adds another 70 mmol/day, ~90% (88 mmol/day) of which is used for protein synthesis (53, 54). Although gut flora produces considerable H_2S, up to 40 μM in the colon, it is effectively oxidized by the epithelium and is not an appreciable source of reduced sulfur (29, 78). The general features of sulfide synthesis and metabolism are shown in FIGURE 2.

H_2S Production

H_2S can be generated via a number of mechanisms from l-homocysteine and l-cysteine via the methionine transsulfuration pathway or from dietary cysteine (15, 58, 160). H_2S can also be formed by reduction of sulfur in persulfides, a process well characterized in protozoans but only recently receiving attention in vertebrates (discussed below). Two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE aka CGL), are found in the cytosol, and the tandem of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST) are found in the cytosol and in the mitochondrial matrix (59, 94, 107). There are also differences in enzyme distribution, CBS predominates in neural and CSE in cardiovascular

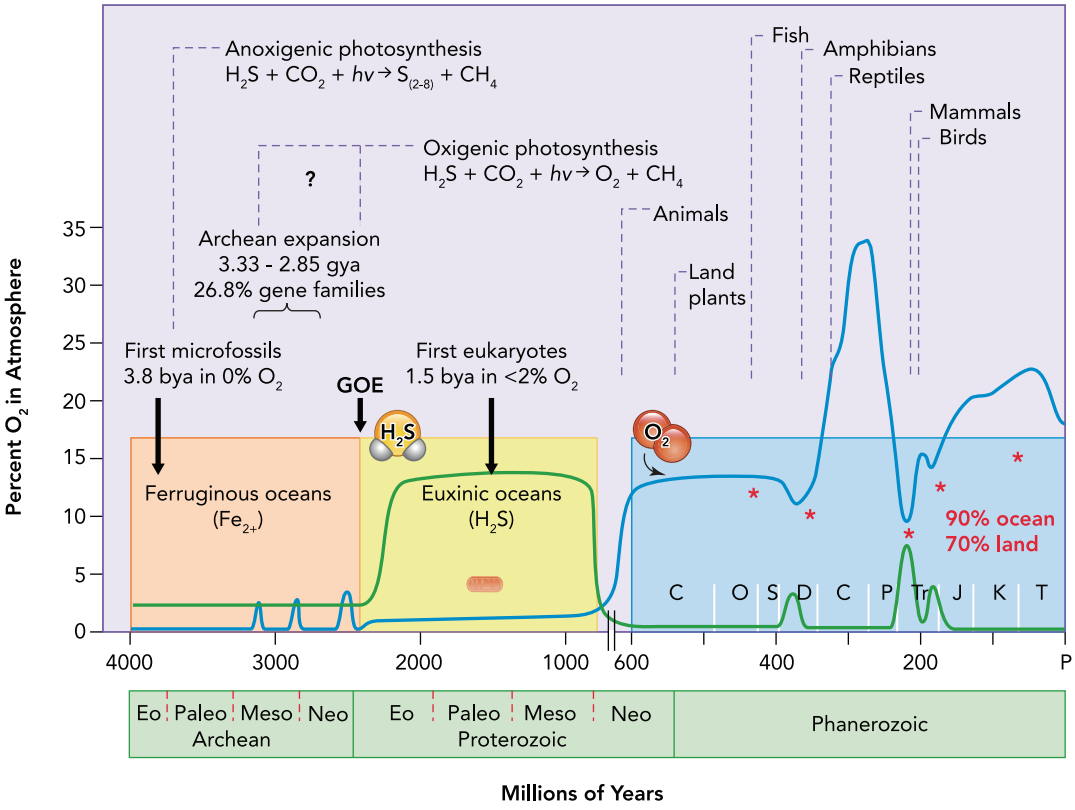

FIGURE 1. **Time line of evolution relative to atmospheric oxygen (O_2, blue line) and oceanic H_2S (orange line)**
Other than possibly a few "whiffs," atmospheric O_2 was essentially nil from the onset of life ~3.8 billion years ago (bya) until the great oxidation event (GOE) 2.3 bya, the latter correlating with a substantial rise in H_2S. Eukaryotes first appeared 1.5 bya in anoxic and sufidic (euxinic) oceans and developed for hundreds of millions of years until O_2 production by oxygenic cyanobacteria and plants oxidized the H_2S and Fe^{2+} ~0.6 bya, essentially eliminating sulfide as an energy source. Mass extinctions (*) were often associated with a fall in ambient O_2 and increase in H_2S, perhaps providing a biological filter for descendants that retained some degree of tolerance to hypoxia and sulfide.

tissues. D-Amino oxidase in brain and kidney peroxisomes may also produce 3-mercaptopyruvate (3-MP) from D-cysteine for delivery to mitochondria (154). A number of studies have shown that H_2S-producing enzymes are regulated by various factors such as calcium (CSE and CAT; Refs. 95, 96), *S*-adenosylmethionine (CBS; Ref. 159), and possibly carbon monoxide (CO) or nitric oxide (NO; although see Ref. 3). Exposed cysteine residues on 3-MST are redox sensitive, and the enzyme is inhibited by oxidative stress (100, 109). Most notable, and alluding to the primordial origin of mitochondria in H_2S metabolism, various types of stress, including hypoxia, translocate CSE from the cytosol to the mitochondria, whereas CBS, which is normally translocated to the mitochondrion for degradation, is no longer catabolized during hypoxia, thus increasing mitochondrial CBS. Both responses, as well as abundant CAT and 3-MST, can synthesize H_2S by taking advantage of the threefold greater cysteine concentration in the mitochondrial matrix compared with the cytosol (32, 166).

However, under normal circumstances, the overall flux of sulfur into the transsulfuration pathway, and hence H_2S production, may be relatively constant. In the presence of oxygen, cysteine dioxygenase (CDO) irreversibly oxidizes cysteine to cysteinesulfinate (and ultimately to hypotaurine or sulfite/sulfate), thereby decreasing S-AA flux through the transsulfuration pathways. CDO activity and expression can increase some 450-fold in response to increased dietary cysteine. Thus as little as 35% of cysteine sulfur is oxidized by CDO in low-cysteine diets, whereas this can increase to 97% when cysteine is in great excess. In this capacity, CDO may serve as a biological "safety valve" setting fairly tight limits on H_2S production (161).

H_2S Catabolism

Of the three transmitters, CO, NO, and H_2S, only the latter is enzymatically inactivated. Chemotrophic and phototrophic microorganisms can oxidize sulfide via a number of different pathways, including sulfur quinone oxidoreductase (SQR), flavocytochrome c sulfide dehydrogenase (Fcc), and the sulfur oxidizing (SOX) pathway, and this can be accomplished aerobically or anaerobically, the latter using nitrate as the electron acceptor (40, 134, 148)

$$HS^- + 1.6NO_{3-} + 0.6H^+ \rightarrow SO_{42-} + 0.8N_2 + 0.8H_2O \quad (7)$$

H_2S can also simply diffuse out of cells, but most evidence suggests that, in eukaryotes, H_2S is inactivated in mitochondria (118). Vertebrates have SQR but neither Fcc nor SOX pathways. Although it is often stated that only prokaryotes use reduced sulfur as electron donors for respiration (148), this is clearly not the case, and a variety of metazoans including invertebrates, fish, birds, and mammals can generate ATP from mitochondrial sulfide oxidation (2, 25, 26, 36, 128, 135, 171, 177).

Vertebrates and invertebrates share common pathways for oxidizing H_2S, although there are still some uncertainties, even in mammals (8, 36, 46, 47, 56, 73, 80, 90, 167). There is general agreement that in the initial step H_2S binds to the SQR enzyme and is oxidized to sulfane sulfur (S) forming persulfide (SQRS-S). This also transfers two electrons via FAD into the quinone pool. These electrons are carried via the electron transport chain to complex III and IV, and the chemiosmotic gradient derived from this drives ATP synthesis. There are differing thoughts on the disposition of the SQR-sulfane sulfur. The Jorns group (56, 90) proposed that sulfane sulfur is first transferred to sulfite ($S_2O_3^{2-}$) forming thiosulfite ($S_2O_3^{2-}$; FIGURE 2, *reaction 1*) and then to glutathione (GSH) forming glutathione persulfide (GSSH). Thiosulfate:glutathione sulfur transferase (TST) supposedly catalyzes the latter step. Although TST has not been identified in mammals, its gene (TSTD1, thiosulfate sulfurtransferase rhodanase-like domain containing 1), homologous to its yeast ortholog RDL1, recently has been identified. TST is not a rhodanase. The mitochondrial sulfur dioxygenase (SDO, aka ETHE1) then oxidizes sulfane sulfur of GSSH to sulfite, consuming O_2 and H_2O in the process. Sulfite can be further oxidized by sulfite oxidase (SO) to sulfate ($S_2O_4^{2-}$), resulting in liberation of $2H^+$ and $2e^-$, the latter transferred to cytochrome c (57) also contributing to ATP production. Alternatively, sulfite can be metabolized by SQR with an additional H_2S to form thiosulfate. Based on kinetic analysis, Libiad et al. (76) proposed an alternative pathway where GSH receives the SQR sulfane sulfur, forming GSSH (FIGURE 2, *reaction 2*). GSSH is then oxidized by SDO (ETHE1) to $S_2O_3^{2-}$, and the GSH recovered. SO_3^{2-} then can be oxidized to $S_2O_4^{2-}$ by SO, or rhodanase (Rhd) can catalyze sulfur transfer from GSSH, producing $S_2O_3^{2-}$. H_2S can also be recovered from $S_2O_3^{2-}$ by endogenous reductants dihydrolipoic acid (DHLA) or thioredoxin (Trx; *reaction 3*; Refs. 94, 120).

In addition to directly stimulating ATP production by donating reducing equivalents to the electron transport chain, H_2S inhibits mitochondrial phosphodiesterase 2A, and the resultant increase in cAMP will further stimulate electron transport (103). ATP production from H_2S has been proposed to balance Krebs cycle-derived electron donors and, by enhancing mitochondrial bioenergetics, helps protect against a variety of stressors (reviewed in Refs. 101, 163). The advent of mito-

chondrial-targeted H_2S-releasing drugs (164) should permit considerable insight into this field.

H_2S Toxicity

The hormetic effect of H_2S is well known; at low concentrations H_2S stimulates O_2 uptake and ATP production, whereas these reactions are inhibited at higher H_2S concentrations through H_2S inhibiton of cytochrome *c*-oxidase (COX). Purified COX is reversibly inhibited by as little as 0.2 μM H_2S, whereas progressively higher concentrations (up to 20–40 μM) are needed to inhibit oxygen consumption by mitochondria and intact cells (1, 8, 17, 102, 129). Thiosulfate is often the excretory product of organisms inhabiting sulfidic and hypoxic environments, since excretion of two sulfur atoms requires only three oxygen atoms, whereas sulfate is normally excreted by animals in normoxic environments (20, 26, 41). SQR activity is generally correlated with increased resistance to H_2S toxicity, and it is increased to offset an increased H_2S load; sulfate-synthesizing enzymes are concomitantly decreased as O_2 availability decreases (33, 42, 44, 55, 74, 97). In acute hypoxia, H_2S may be detoxified by reversing electron flow and reducing fumarate to succinate (36, 41). This has been pro-

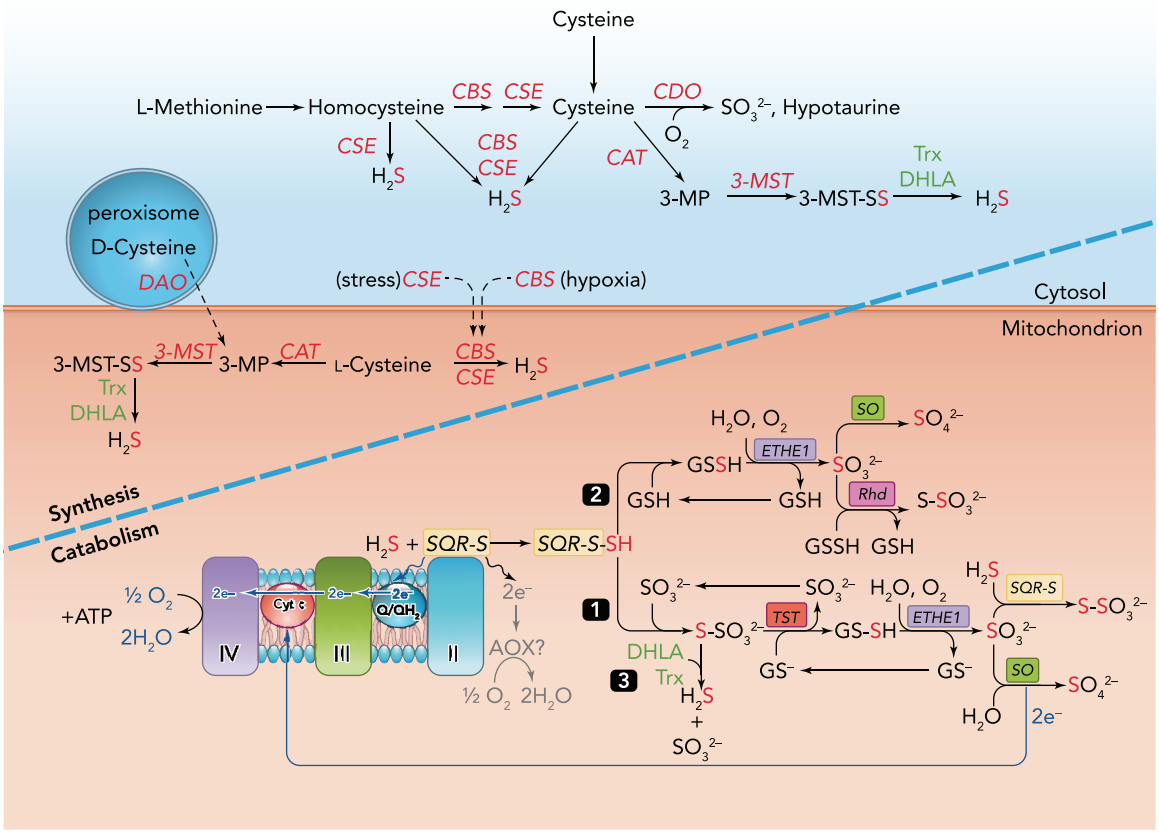

FIGURE 2. **Pathways for H_2S production and catabolism in vertebrates**

H_2S synthesis: in the cytosolic transsulfuration pathway, homocysteine generated from methionine can directly, or in combination with L-cysteine, produce H_2S catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). H_2S can also be produced directly from dietary cysteine. Cysteine dioxygenase (CDO) maintains intracellular cysteine concentrations, and potentially H_2S production, relatively constant by oxidizing excess cysteine to cysteine sulfonate, which then becomes sulfite ($S_2O_3^{2-}$) and hypotaurine. CBS and CSE can also be translated into the mitochondria to take advantage of threefold higher cysteine concentrations in the matrix. Cysteine aminotransferase (CAT) catalyzes the formation of 3-mercaptopyruvate from cysteine, which then forms a persulfide with the enzyme 3-mercaptopyruvate sulfur transferase (3-MST) in both cytosol and mitochondria. H_2S can presumably be released from 3-MST-SH by another reductant such as thioredoxin (Trx) or dihydrolipoic acid (DHLA). D-Amino acid oxidase (DAO) in brain and kidney peroxisomes can also generate 3-MP from d-cysteine. *H_2S catabolism*: H_2S binds to the enzyme sulfur quinone oxidoreductase (SQR), forming a persulfide (SQRS-S), in the process transferring two electrons via a quinone into the electron transport chain. These electrons ultimately are delivered to oxygen, and ATP is produced. In *path 1*, the sulfane sulfur is first transferred to the mobile carrier sulfite ($S_2O_3^{2-}$), forming thiosulfate ($S_2O_3^{2-}$), and then to glutathione (GSH) by thiosulfate sulfur transferase (TST), forming glutathione persulfide (GS-SH). Mitochondrial sulfur dioxygenase (ETHE1) oxidizes GS-SH to sulfite, which can then be further oxidized by sulfite oxidase (SO) to sulfate (SO_4^{2-}) producing electrons that are delivered to cytochrome c (Cyt c) or receive another H_2S and form thiosulfate. *Pathway 2* is similar except that GSH is the initial mobile carrier and rhodanase (Rhd) catalyzes formation of thiosulfate from sulfite and GSSH. H_2S can also be regenerated from thiosulfate by endogenous reductants dihydrolipoic acid (DHLA) and thioredoxin (Trx). An alternative oxidase (AOX) that accepts electrons from SQR but is not coupled to ATP production is found in invertebrates.

posed to protect cells by sustaining ATP production (32), although direct evidence for such an event is lacking, and fumarate availability may be limiting. H_2S toxicity also may be mitigated by increasing mitochondrial dehydroascorbic acid (45).

H_2S and Sulfur Signaling

Numerous homeostatic functions have been proposed for H_2S, including cytoprotection, anti-inflammation, neuromodulation, and cardiovascular function (reviewed in Refs. 14, 68, 133, 174). These studies are based largely on the effects of exogenous H_2S administration or after manipulation of H_2S-metabolizing enzymes. How endogenous H_2S is regulated is unclear. H_2S also has been proposed to be an oxygen sensor (117). In this instance, it is clear that H_2S concentration can be tightly regulated by the balance between constitutive H_2S production through transsulfuration and the amount of oxygen available for its metabolism. The protective effects of H_2S in a variety of models of ischemia (133) likely reflect a similar mode of oxygen-dependent H_2S metabolism.

Four mechanisms of H_2S signaling have been identified thus far. *1*) Although supraphysiological concentrations of H_2S inhibit mitochondrial COX, lower (and presumably physiological) concentrations contribute to energy production and mitochondrial stability (8, 36, 101, 163). Separating physiological from toxicological effects is an ongoing difficulty. *2*) Completely reduced H_2S sulfur (-2) can act as a reductant, and this appears to be a highly specific process for certain disulfides (170). Further identification of these disulfides and their proximity to H_2S production should greatly enhance our understanding of H_2S signaling. *3*) Dissolved H_2S or HS^- can coordinate with or reduce iron in heme proteins. This has recently been described in a variety of complex reactions that regulate activity of heme peroxidases, such as myeloperoxidase and catalase (110, 124). *4*) Perhaps the most interesting signaling mechanism is sulfhydration (more appropriately termed sulfuration). Two-electron oxidation of either H_2S or cysteine sulfur (or a one-electron oxidation of both) forms sulfane sulfur, S^0 (168), which can react with a variety of other sulfur atoms in proteins and low molecular weight molecules to form persulfides and polysulfides. These are described in the following section.

Polysulfide Production and Metabolism: the "Next Frontier"?

Evidence is accumulating that polysulfides (RS_nR, RS_nH, H_2S_n; $n > 2$) or persulfides ($n = 2$) may be the actual mediators of sulfide signaling (99, 110, 122, 125). These readily interact with regulatory protein cysteine sulfur and nitrogenous signaling species through a variety of mechanisms and can act as either an oxidant or a reductant (18, 38, 66, 67, 105, 111, 168, 169). It has been suggested that as much as 25% of protein cysteines in mammalian cells may have a sulfane sulfur associated with it (106).

Comparatively little is known about polysulfide metabolism in vertebrates, and most attention has focused on its role in H_2S production and subsequent signaling. In the canonical pathway (FIGURE 2), cysteine metabolism by CAT and 3-MST generates the 3-MST persulfide (3-MST-S). Addition of a reductant such as thioredoxin or dihydrolipoic acid then releases H_2S from the persulfide (69, 94, 108, 176). The sulfane sulfur (S) can also be transferred to another mobile thiol such as cysteine, homocysteine, or glutathionine, e.g., 3-MST-S + RSH $\rightarrow$ 3-MST + RS-SH (176), and wend its way along to less mobile protein thiols (30, 122). Recently, Kimura's group has shown that H_2S_3 can be formed directly from 3-MP by 3-MST and rhodanase in mammalian cells (70).

CSE and CBS catalyze the formation of a variety of cysteine hydropolysulfides (CysSSH, CysSSSH, and CysSSSSH) and, secondarily, polysulfides (CysSSSCys, CysSSSSCys, CysSSSSSCys) from cystine (CysSSCys) in mammalian cells (FIGURE 3A; Ref. 52). Cystine is far more prevalent than cysteine or methionine in the oxidized extracellular environment, and it is readily transported into cells by the cystine/glutamate antiporter, system X_c^- (11), or possibly the sodium-coupled neutral amino acid transporter (AT2; Ref. 52). This process can provide substantial sulfane sulfur in an intracellular store that may then be transferred to glutathione (GS_nH and GS_nG; $n = 2$–4) and act as an intracellular reductant or intracellular signal (52). Unlike H_2S, where intracellular concentrations are expected to be in the low nanomolar range (121), high polysulfide concentrations can be achieved; glutathione persulfide has been estimated to exceed 100 μM (52).

Recycling polysulfides for H_2S or energy production has yet to be examined in vertebrates, but it has been described in some prokaryotes, most notably phototropic (green and purple) sulfur bacteria (FIGURE 3B; Refs. 31, 37). Sulfur generated in anoxigenic photosynthesis (*Eq. 4*) is stored in intracellular or extracellular sulfur globules. Interestingly, cyclization and precipitation as elemental sulfur (S_8) is inhibited, and sulfur is retained as long ($n > 3$ and possibly up to $n > 10^5$), linear, and stable polymers. These can be further oxidized or reduced back to H_2S if environmental H_2S availability falls. This regeneration of H_2S as an electron donor may be the antecedent of eukaryotic sulfur

cycling important for mitochondrial integrity or redox signaling.

Polysulfides may have another unappreciated link with evolution and our current concept of both toxicity and signaling with reactive oxygen species (ROS). Stepwise one-electron oxidation of H_2S (HS^-) initially produces a thiyl radical ($HS^{\cdot-}$; FIGURE 1C). Two of these can combine to produce hydrogen persulfide (H_2S_2), which then can be oxidized to a persulfide radical ($S_2^{\cdot-}$) and then to elemental sulfur (S_n). These intermediates, reactive sulfide species (RSS), are surprisingly chemically and biochemically similar to the ROS intermediates in one-electron reduction of oxygen (FIGURE 3D) or one-electron oxidation of water. However, RSS have been around since life originated and were probably very prevalent in early anoxigenic photosynthesis. Conversely, ROS only became an appreciable physiological problem after oxigenic photosynthesis caused oxygen to be formed some 600 million years ago. Hydrogen peroxide (H_2O_2) has garnered most attention as a signaling ROS because of its relative stability, membrane permeability, and ability to selectively react with protein thiols (175). Hydrogen persulfide (H_2S_2) shares many of these characteristics with hydrogen peroxide but appears even more reactive than peroxide in inactivating the lipid phosphatase PTEN (38). It is quite likely that some of the perceived ROS signaling may in fact be RSS signaling. Our laboratory (DeLeon ER, Gao Y, Huang E, Arrif M, Arora N, Divietro A, Olson KR, unpublished observations) recently found that a number of methods historically used to measure ROS, including redox-sensitive green fluorescent protein (roGFP), 2′,7′-dihydrodichlorofluorescein (DCF), MitoSox Red, Amplex Red, and H_2O_2 amperometric electrodes, are as, or often more, sensitive to RSS than they are to ROS. How these findings impact our understanding of cellular oxidants, antioxidants, and redox signaling remains to be determined. Sorting this out is the "next frontier" in sulfide biology. ■

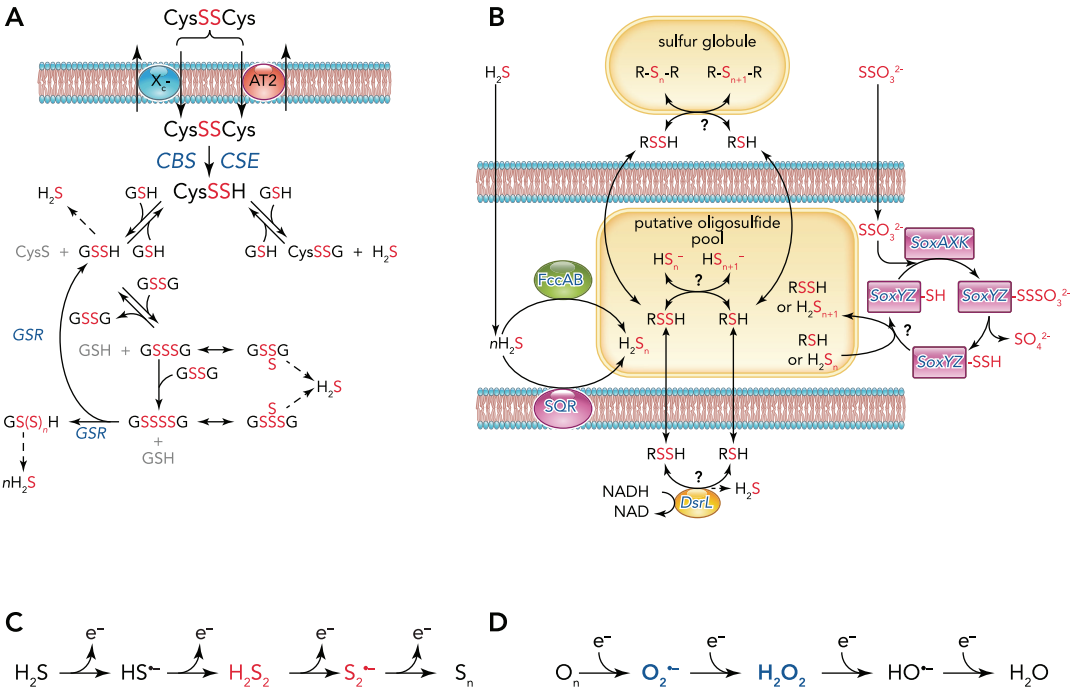

FIGURE 3. **Polysulfide shuttling in mammals and green sulfur bacteria, and similarities between reactive sulfide species and reactive oxygen species**

A: in mammals, cystine (CysS-SCys), abundant in plasma and extracellular fluid, is taken up by cells via the cystine/glutamate antiporter (system X_c^-) or via the sodium-coupled neutral amino acid transporter (AT2). Cytosolic CBS and CSE then catalyze formation of cysteine (Cys) hydrosulfides and polysulfides [CysS-S$_{(n)}$H and CysS-S$_{(n)}$Cys, respectively], and Cys can be exchanged for glutathione (GSH or G). H_2S can be regenerated from the hydrosulfides and polysulfides by two electron reductants. Image is modified from Ref. 52 and is used with permission from *Proc Natl Acad Sci USA*. *B*: generic mechanisms of polysulfide (PS) shuttling by phototropic green and purple sulfur bacteria. H_2S is taken up and oxidized by sulfur quinione:oxidoreductase (SQR) similar to eukaryotes, or flavocytochrome *c* (FccAB), and ultimately stored in an intracellular (not shown) or extracellular globule as linear polysulfides that can exceed 10^5 sulfur molecules. The sulfide oxidation (SOX) pathway metabolizes thiosulfate via Sox enzymes (SoxAXK and SoxYZ) that also form polysulfides. Stored polysulfides can be recovered during low H_2S and H_2S regenerated by dissimilatory sulfide reductases (DsrL) using electrons from NADH. Image is modified from Ref. 37 and is used with permission from *Front Microbiol*. *C*: stepwise one-electron oxidation of H_2S forms the thiyl radical ($HS^{\cdot}$), hydrogen persulfide (H_2S_2), persulfide radical ($S_2^{\cdot-}$), and elmental sulfur (S_n). *D*: stepwise one-electron reduction of O_2 forms superoxide ($O_2^{\cdot-}$), hydrogen peoxide (H_2O_2), hydroxyl radical ($O_2^{\cdot-}$), and water. Biologically important reactive oxygen species (in blue) are homologous to reactive sulfide species (in red).

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