Endogenous Production of H₂S in the Gastrointestinal Tract: Still in Search of a Physiologic Function

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Abstract

Hydrogen sulfide (H₂S) has long been associated with the gastrointestinal tract, especially the bacteria-derived H₂S present in flatus. Along with evidence from other organ systems, the finding that gastrointestinal tissues are capable of endogenous production of H₂S has led to the hypothesis that H₂S is an endogenous gaseous signaling molecule. In this review, the criteria of gasotransmitters are reexamined, and evidence from the literature regarding H₂S as a gaseous signaling molecule is discussed. H₂S is produced enzymatically by gastrointestinal tissues, but evidence is lacking on whether H₂S production is regulated. H₂S causes well-defined physiologic effects in gastrointestinal tissues, but evidence for a receptor for H₂S is lacking. H₂S is inactivated through enzymatic oxidation, but evidence is lacking on whether manipulating H₂S oxidation alters endogenous cell signaling. Remaining questions regarding the role of H₂S as a gaseous signaling molecule in the gastrointestinal tract suggest that H₂S currently remains a molecule in search of a physiologic function. *Antioxid. Redox Signal.* 12, 1135–1146.

A LONG-STANDING ASSOCIATION exists of hydrogen sulfide (H₂S) with the gastrointestinal tract. After the initial systematic description of the physical properties of H₂S, chemists of the late 18th and early 19th centuries, by using the intestines of dead and decaying animals, identified H₂S gas as a major constituent of the process of putrefaction. Lehmann (33) described the culmination of several studies in the first half of the 19th century that documented H₂S as a constituent of intestinal gas before postmortem putrefaction. The small amount of H₂S in normal flatus made early chemists consider digestion in the large intestine as the same chemical process as putrefaction, which led many physicians, including Dr. John Kellogg, creator of Kellogg's Corn Flakes, to prescribe colonic cleansing or hydrotherapy to maintain, perhaps inappropriately, colonic health. At the advent of microbiology, Gayon (18) described the ability of isolated bacteria to generate H₂S from albuminous material. His work ushered in a flurry of taxonomic and biochemical studies in the late 19th century and the first half of the 20th century regarding the enzymatic production of H₂S by commensal bacteria.

Whereas the vast majority of early scientific studies regarding H₂S and the gastrointestinal tract dealt with H₂S of bacterial origin, recent work on H₂S is focused on the role of endogenously produced H₂S from nonbacterial sources. This recent activity is due to the emergence of H₂S as an endogenous signaling molecule. A mere 13 years ago, Abe and

Kimura (1) described the enzymatic mechanism of H₂S production in the brain and the biologic effects of and cellular targets for H₂S. Their work began what was to become an exponential increase in studies related to the cell-signaling properties of H₂S. The disciplines of chemistry, pharmacology, molecular biology, and physiology have converged to support the hypothesis that H₂S is an endogenous molecule that regulates physiologic and pathophysiologic processes. The intention of this article is to review critically the literature that supports the role of H₂S as a gaseous signal molecule in the gastrointestinal tract. In some areas of this review, data from other organ systems are included to highlight areas that require attention in the gastrointestinal tract. To set the stage for this review, we first provide our views on the use of the term gasotransmitter and follow with a reevaluation of the criteria being considered for H₂S as a gaseous signaling molecule.

Gasotransmitter Criteria Revisited

The term gasotransmitter implies that the candidate gaseous molecule is synthesized and released into the extracellular spaces to transmit a signal by acting on another cell. When released from a neuron, a candidate gaseous molecule can be considered to function as a neurotransmitter, by following classic definition for a neurotransmitter (70). The

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elevation of intracellular calcium and induction of calcium waves in astrocytes and microglia in the central nervous system (CNS) by NaHS suggests that endogenous H₂S functions *in vivo* as a neurotransmitter. When H₂S is released from nonneuronal cell types, including intraluminal colonic sulfur-reducing bacteria, use of the term transmitter to describe the action of H₂S also is appropriate. For the past several years, a substantial amount of data has been acquired to support the hypothesis that H₂S can also act on targets within the cell in which it is produced. In this regard, H₂S functions as an intracellular signaling molecule. In this review, we use the term gaseous signaling molecule to include both putative transmitter and intracellular signaling roles of H₂S.

In 2002, Rui Wang (67) set forth the following five criteria for a molecule to be classified as a gasotransmitter and included evidence demonstrating that H₂S met these criteria. Gasotransmitters must

1. be small molecules of gas,
2. be freely permeable to membranes,
3. be endogenously and enzymatically generated in a regulated manner,
4. have well-defined specific functions at physiologically relevant concentrations, and
5. act at specific cellular targets.

These criteria have been very helpful to define the focus of the field of H₂S biology. We propose that they can now be updated.

With regard to the first criterion, it is not necessary to constrain the size of a putative gasotransmitter. We propose removing the word "small." Although NO, CO, and H₂S, are freely diffusible through membranes, it is unclear why putative gasotransmitters must fulfill the second criterion. Gaseous molecules that are polar and not freely diffusible would not meet the second criterion but may still be considered gaseous signal molecules. Therefore, we propose eliminating this criterion. The third criterion, a regulated enzymatic pathway for the production of the gas, is reasonable. Although Wang's fourth criterion also is reasonable, more consideration should be given to what are physiologically relevant concentrations. In the case of H₂S, a number of original research articles and reviews (compiled by 71) have stated that H₂S is an endogenous signal molecule, because resting tissue and plasma levels of H₂S are similar to the effective concentration of exogenous NaHS. Most *in vitro* studies of the influence NaHS of on tissue function have required a tissue bath concentration of 100 μ M to alter tissue activity appreciatively. Although several studies have identified tissue concentrations in the micromolar range, these measurements have been called into question (63, 71), and a recent report indicates that the free H₂S concentration in tissue (brain and liver) appears to be only $\sim$ 20 nM (16), roughly 1/5,000 of the requisite bath concentration. Because inhalation of 2,240 ppm H₂S (equivalent to 100 μ M H₂S) is lethal to animals, it becomes important to determine what actions of H₂S/HS[−] observed in *in vitro* studies reflect toxicity to tissue *versus* physiologically relevant signaling of tissue activity. Several possible explanations exist to explain how H₂S could serve as a gaseous signal molecule, despite the large discrepancy between the concentrations of H₂S in tissue *versus* those needed for alteration of tissue function, *in vitro*. First, diffusion of H₂S/HS[−] into even relatively small pieces of tis-

sue (*i.e.*, 50 mg) is relatively slow. Most tissues have an effective means of metabolizing H₂S, so a gradient of H₂S concentration will develop in the tissue, with only the most superficial layer having an H₂S concentration equal to that of the bath, whereas cells in the interior of the tissue would be exposed to much lower, even trivial, concentrations of H₂S. Second, because H₂S is both produced and consumed by cells, concentrations of H₂S/HS[−] at localized sites of release in the cell may be much higher than those observed in whole tissue. As a result, receptor-like molecules located in proximity to the release site could be exposed to high H₂S concentrations, whereas the overall tissue concentrations remain low.

The complexity of relating the concentration of H₂S in the bathing solution to a physiologically relevant concentration is an echo of earlier research on acetylcholine as a neurotransmitter. Although acetylcholine was discovered in the 1920s, it was not possible to measure acetylcholine levels accurately prior to 1965, and tissue levels were determined to be relatively high. Today, extremely sensitive methods are available for its detection. The level of acetylcholine in the cerebrospinal fluid of humans is $\sim$ 0.3 nM (74), whereas the half-maximal effective concentration (EC₅₀) value for acetylcholine activation of $\alpha_4\beta_3$ postsynaptic nicotinic acetylcholine receptors is $\sim$ 75 μ M (47). Yet, the validity of acetylcholine as a neurotransmitter in the CNS is not questioned. It is safe to assume that, as in the early days of acetylcholine biology, it has not yet been possible to measure accurately the concentration of "physiologically relevant" H₂S. The development of fluorescent markers to determine the spatiotemporal concentrations of H₂S will likely be a key advance in the future. We suggest that it is important to include within the language of the fourth criterion that physiologic effects occur on exogenous application of physiologically relevant concentrations of the candidate molecule, such that it mimics the effect of the proposed endogenous signaling pathway.

We also propose that the fifth criterion regarding specific cellular targets be modified. A drug is classified as acting at a receptor only if it can be competitively antagonized by a related molecule (26). Competitive antagonism remains a key criterion of neurotransmitters and signaling molecules, as it is the accepted method of determining that a specific receptor exists for the drug. We propose adding the concept of competitive antagonism to the fifth criterion, despite the lack of experimental evidence for this criterion being fulfilled for H₂S (see later).

Finally, a key criterion in the case of H₂S acting as a neurotransmitter (70) or signaling molecule that is absent from Wang's list is a mechanism for inactivation. An important aspect of the information encoded by a signal molecule, especially a neurotransmitter, is a temporal signal. Regulated control of the duration of cell signaling is as dependent on a specific mechanism of signal termination as it is on the mechanisms of regulated synthesis and release. Few signal molecules rely on passive diffusion, and thus, dilution of the signal molecule as a form of inactivation. Rather, most signal molecules use transporter uptake or enzymatic degradation to shorten the duration of the response. Uptake and degradation help protect the cells receiving the signal from overstimulation, which is often fatal to the cell, and help keep systemic concentrations of the active molecule from reaching high concentrations. We propose adding this criterion to the list and suggest that the rapid oxidation of H₂S by tissues be considered in future studies of H₂S physiology (see later).

We propose that the criteria for a gasotransmitter be revised such that a gaseous signaling molecule must

1. be a gas,
2. be endogenously and enzymatically generated in a regulated manner,
3. with exogenous application, cause a well-defined physiologic effect at physiologically relevant concentrations that mimics the effect of the endogenously produced H_2S on tissue activity,
4. act at specific cellular targets, as demonstrated by competitive antagonism, and
5. employ a specific mechanism of inactivation.

The remainder of this review is devoted to highlighting the current evidence that H_2S fulfills some of these criteria, with an emphasis on evidence from the gastrointestinal tract, and highlighting areas of research that require attention for these criteria to be met. Because the third and fourth criteria overlap, they are considered together.

The First Criterion: H_2S is a Gas

The physical properties of H_2S gas are well described (4, 45). H_2S exists as a gas because its boiling point is approximately -60°C . Aqueous solutions absorb H_2S such that, at equilibrium, H_2S in the aqueous phase is ~ 2.2 times the concentration of the gas phase at 37°C . Increased temperatures slightly decrease the solubility of H_2S , but changing pressure has little effect on this equilibrium. In aqueous solution, the pK_a of the dissociation of H_2S to HS^- is 7.02 at 25°C . Because $\text{pH} = \text{pK}_a + \log_{10} ([\text{HS}^-]/[\text{H}_2\text{S}])$, the ratio of $[\text{HS}^-]/[\text{H}_2\text{S}]$ at pH 7.4 must have an antilog equal to 0.38 ($7.4 - 7.02$); thus, the ratio of $[\text{HS}^-]:[\text{H}_2\text{S}]$ is 2.4 at pH 7.4. Although measurements of intracellular pH have been quite variable, many such measurements have indicated that intracellular pH is ~ 7.0 , which would result in roughly equal intracellular concentrations of H_2S and HS^- . However, because the pK_a of this dissociation decreases with increased temperature, the ratio of $[\text{HS}^-]:[\text{H}_2\text{S}]$ is higher at 37°C (12). This discussion underscores the concept that dynamic changes in temperature and pH can dramatically affect the concentration of dissolved H_2S in physiologic preparations. Although HS^- can dissociate to H^+ and sulfide (S^{2-}), the pK_a of this dissociation is ~ 12 ; thus, negligible S^{2-} exists at physiologic pH.

Although it has been suggested that the active form of H_2S *in vivo* may be HS^- (12), which is not a gas, the source of endogenous HS^- is the dissociation of enzymatically produced H_2S . In the literature, the use of the term H_2S usually refers to the sum of free H_2S and HS^- , as is the case in this review. H_2S is lipid soluble and not constrained by cellular membranes, but due to its partial dissociation, the lipophilic plasma membrane is less permeable to H_2S than it is to NO and CO.

The Second Criterion: Endogenous Production of H_2S

H₂S-producing enzymes

General interdisciplinary view of H_2S -producing enzymes. The original demonstration of biologic production of H_2S by bacteria (18) began nearly a century and half of comprehensive studies describing the biochemistry of H_2S production. H_2S is a constituent of intestinal gas and is the principal by-

product of sulfur-reducing bacteria (*desulfovibrio*) in the mammalian colon. H_2S concentrations in excess of 1,000 ppm have been measured in gas samples obtained from the rat cecum (59). H_2S and methyl mercaptan also are produced by the flagellate bacterium *Helicobacter pylori* and may account in part for halitosis (31).

As in bacteria, H_2S gas is formed naturally in vertebrates, including humans, through the activity of cystathionine β synthase (CBS), cystathionine γ lyase (CSE), both 2-pyridoxal-5'-phosphate (PLP) dependent, and 3-mercaptopyruvate sulfurtransferase (3MST) (23, 56, 57), a non-PLP-dependent enzyme. CBS and CSE may function as cellular redox sensors, increasing H_2S generation in response to intracellular oxidant load (38). Physiologic acidification is required for 3MST activity (23).

Although several compounds are used to inhibit H_2S -synthesizing enzymes, a recent call was made for the development of new pharmacologic tools with improved selectivity and specificity (62). Aminooxyacetate and hydroxylamine are used to inhibit CBS. As both compounds target the PLP-binding domain of CBS, they also are effective inhibitors of other PLP-dependent enzymes, including CSE and aminotransferases. DL-Propargylglycine and β -cyano-L-alanine are used as irreversible and reversible inhibitors, respectively, of CSE and demonstrate a certain degree of specificity. Hydrogen peroxide and tetrathionate inhibit 3MST by interfering with the catalytic cysteine residue, similar to the effect these compounds have on all enzymes that use cysteines in the catalytic site. The 2- and 3-mercaptopyruvate acids are uncompetitive and noncompetitive inhibitors of 3MST, respectively.

The human CBS gene is located on chromosome 21 (21q22.3) (30) and encodes several splice variants (19). An autosomally inherited recessive deficiency of CBS through multiple genetic polymorphisms results in homocysteinuria (29). CBS activity and hence H_2S production is increased in Down syndrome (25). The structure of a truncated form of human CBS has been solved (44). The gene that encodes human CSE (CTH) is located on chromosome 1 (1p31.1), encoding at least two splice variants (37). Polymorphisms in the CSE gene are associated with cystathionuria (66). The structure of CSE, bound to its inhibitor propargylglycine, has been recently solved (Fig. 1) (28, 61). This advance is likely to result in the development of new and more-selective enzyme inhibitors. The gene that encodes human 3MST (MPST), also known as liver rhodanese and thiosulfate sulfurtransferase 2 (TST2), is located on chromosome 22 (22q11.2) and encodes at least three splice variants (6, 50). This gene should not be confused with thiosulfate sulfurtransferase (TST) or rhodanese, which is immediately adjacent on chromosome 22 (22q13.1) but encodes a distinct enzyme (2). Polymorphisms in 3MST may underlie mercaptolactate-cysteine disulfiduria (6).

H_2S -producing enzymes in the gastrointestinal tract. Transcripts encoding both CSE and CBS have been identified in gastrointestinal tissues in rats (13) and mice (36). Both CSE and CBS are expressed, as determined by immunoreactivity, in the mouse colonic mucosa, whereas only CSE is expressed in the external muscle layers of the colon, with the highest level of immunoreactivity in enteric neurons (36). The very low expression levels of mRNA encoding CBS in the external muscle layers, including the myenteric plexus, suggests that CSE activity predominates in the mouse colon. The absence of

FIG. 1. Ribbon diagram of human CSE monomer derived from x-ray crystallography data. Pyroxidyl-5-phosphate (PLP) is shown in black, and the PLF-binding domain is shown in the darker shade of gray. The smaller domain of CSE is shown in light gray. Native CSE likely exists as a tetramer. Reproduced with permission from (61).

CBS in mouse colonic enteric neurons is at variance with a previous report that both CBS and CSE are present in guinea pig and human enteric neurons of the colon (55). This difference may reflect species differences. The use of specific inhibitors of CSE and CBS and measurement of endogenous production of H_2S gas may be helpful in determining the relative contribution of the two PLP-dependent enzymes in the same organ across species.

H₂S production and release

General interdisciplinary view of H_2S production. Historically, the presence of H_2S gas has been qualitatively measured by its ability to discolor metals. The ability of metals to absorb H_2S has been used until the present day to “trap” sulfide as a stable solid (58). On acidification of stable and soluble metal-sulfide, H_2S gas is released and can be measured quantitatively with numerous techniques. Perhaps the most common method of H_2S gas measurement is the methylene blue assay, the history and use of which is described by Jacobs and colleagues (24). H_2S reacts with *N,N*-dimethyl-*p*-phenylenediamine and ferric chloride in a strongly acidic solution to produce methylene blue [3,7-bis (dimethylamino) phenazathionium chloride], which can be quantitatively measured with colorimetric or spectrophotometric analysis.

New methods are being developed to assay H_2S gas production. Polarographic technology is being used to demonstrate H_2S release from sulfide-containing compounds (11). Electrodes sensitive to H_2S are being used in a variety of tissues, and the results support the concept that previous reports of H_2S production greatly overestimate H_2S levels (71). Gas chromatography remains an accurate and reliable method of measuring H_2S , but methods used to collect the gas sample have recently been scrutinized. H_2S gas is highly labile, and samples degrade rapidly, even in sealed storage containers. Furne and colleagues (16) recently reported that when tissue was rapidly homogenized, and the gas released from the tissue was assayed immediately and directly for H_2S , the tissue

contained only $\sim 10^{-8}$ M as compared with previous estimates of 10^{-5} M (35). Furne and colleagues (16) suggested that the discrepancy might arise from the addition of cysteine (up to 10 mM) in the tissue homogenates to stimulate H_2S production, which was not added in their analysis. Cysteine in solution is capable of nonenzymatic decomposition to H_2S that can be readily detected by smelling a vial of dissolved cysteine.

Several studies demonstrated that H_2S production is altered by experimental manipulation. By using the enzymatic assay of acid-induced release of H_2S in the presence of cysteine, H_2S production *in vivo* is increased after ischemia (77) and decreased in diabetic mice (7). In cell culture, incubation of H_2S -producing cells with glucose (76) or the NO-releasing compound sodium nitroprusside (79) increases H_2S production. It is of interest to note that because these measurements were obtained through enzymatic assays with excess cysteine, these measurements may have simply reflected increased expression of the enzymes rather than a change in their production levels. In a recent and elegant study, the constitutive production of H_2S was found to be offset by mitochondrial oxidation (49). As tissue O_2 decreases, H_2S oxidation declines, thereby increasing the cellular concentration of H_2S to exert its physiologic effect.

H_2S production in the gastrointestinal tract. Several groups have measured the enzymatic production of H_2S in the gastrointestinal tract (13, 22, 36). Unlike H_2S -production assays in other regions of the body, the presence of sulfate-reducing commensal bacteria within the lumen of the gastrointestinal tract can contribute to the amount of H_2S that is measured. To eliminate the contribution of luminal bacteria, we developed a method to measure quantitatively the endogenous generation and release of H_2S gas in intact and living muscle layers of the mouse colon, containing the myenteric plexus dissected away from the mucosa, without contamination of luminal bacteria (36). The rate of H_2S gas production and release from intact tissue averaged 0.45 pmol/min/mg tissue and was inhibited when the tissue was treated simultaneously with CSE and CBS inhibitors. The level of nonbacterial H_2S production in intact tissue is at least an order of magnitude lower than the level of H_2S production in the same tissue after it was homogenized (36) and considerably lower than previous reports for homogenized tissues (13, 22). These observations suggest that either homogenization disrupts the normal proximity of the enzymes that produce H_2S gas to the enzymes responsible for its degradations or that the relatively high levels of H_2S previously reported were not of free H_2S gas but instead of H_2S released from acid-labile and bound cytoplasmic stores (23), or both. As described earlier, current methods being used to measure H_2S rely on the presence of exogenous cysteine. By using the method developed by Furne and colleagues (16) without the addition of cysteine, we recently determined that mouse colonic muscle tissue, obtained aseptically without luminal bacteria or the addition of cysteine, contains ~ 60 nM H_2S (Fig. 2).

Because CSE is highly expressed in enteric neurons, we conducted experiments to test whether H_2S production and release could be altered by electrical field stimulation (EFS) by using standard protocols for nerve stimulation and our method of measuring H_2S production from living tissue (36) (Fig. 3). EFS delivered by bipolar platinum-ring electrodes

FIG. 2. Facsimile of representative elution peaks of H_2S by using gas chromatography and sulfur fluorescence detection, demonstrating that the external muscle layers of the colon contain H_2S without supplementation with cysteine or other stimulation. Whereas room air contained ~ 8 ppb H_2S , 3 ml of room air mixed vigorously with colonic tissue contained ~ 13 ppb H_2S . With the mass of the tissue and assumed equilibrations between dissolved and gaseous H_2S and H_2S and HS^- , the calculated concentration of H_2S in the tissue was ~ 60 nM.

eliminated H_2S gas in the samples. However, subsequent experiments using a solution of NaHS without tissue revealed that H_2S was eliminated in an electrochemical manner. In retrospect, the electrochemical properties of H_2S are well known, as metal anodes effectively oxidize H_2S to sulfate (45), which forms the basis for the electrochemical detection of H_2S (71). We are currently investigating other mechanisms of nerve stimulation to determine whether H_2S gas production and release in the colon wall can be regulated by enteric neuronal activity.

A strong potential source of acute H_2S regulation is the dynamic availability of free cysteine. Cysteine is "stored" in proteins, sulfomucins, and in glutathione, such that cytosolic levels are normally quite low. Cysteine availability appears to be responsible for changes in the levels of H_2S in flatus. Lack of suitable substrate in the colonic chyme sulfomucins can limit the amount of H_2S produced (59). The rate-limiting step in H_2S production may be the dynamic and regulated release of free cysteine.

Summary of the second criterion. Collectively, the literature supports the view that H_2S is produced endogenously in the gastrointestinal tract in partial fulfillment of the second criterion. Potential regulation of endogenous H_2S production remains a question that requires further investigation. Future studies that test the dynamic regulation of H_2S production by intestinal tissues are likely to reveal mechanisms that can be applied to other organ systems.

FIG. 3. With the methods of Linden and colleagues (36), H_2S produced in 1 ml of normal Krebs' solution containing 10 mM cysteine and either intact living colonic muscle (14 to 20 mg) (left) or 500 μM NaHS (right) were subjected to electrical field stimulation (EFS) by bipolar platinum ring electrodes separated by 0.5 cm (trains of 0.5-ms pulses, 10 Hz for 30 s, every 5 min for 30 min). EFS likely oxidized all H_2S in solution to sulfate at the anode. Data are expressed as mean $\pm$ SEM; $n = 3$; $*p < 0.05$; ANOVA or t test compared with unstimulated.

The Third and Fourth Criteria: Physiologic Effects of H_2S at Specific Cellular Targets

General interdisciplinary view of the action of H_2S

The well-described activation of soluble guanylyl cyclase (sGC) by epithelium-derived relaxing factor (EDRF), and the subsequent demonstration that NO reversibly and competitively binds the heme moiety of sGC led to general acceptance of NO as a gasotransmitter because cytosolic sGC acts like a classic membrane-bound receptor for the endogenous ligand NO (5). Unlike NO, no well-described orphan receptor is waiting for H_2S to fulfill the role of an endogenous ligand. The search for potential receptors for H_2S has used pharmacologic approaches that implicate potential interacting proteins. However, specific receptors for H_2S have not been identified. Cytochrome *c* oxidase, the molecular target for H_2S -mediated toxicity, remains the only protein demonstrating H_2S binding in a reversible fashion (48). The lack of demonstrable H_2S -binding sites and competitive antagonism (a criterion of a gaseous signal molecule set forth in a previous section of this review) has led to much speculation regarding the identity of receptors for H_2S , but to little direct evidence for ligand-receptor interaction.

Putative molecular targets appear to be both tissue and species dependent. H_2S potentiates NMDA receptors during repetitive nerve stimulation (1) through cAMP-dependent pathways, opens K_{ATP} channels (17, 80), voltage-gated potassium channels (17), apamin-sensitive potassium channels (17), $\text{Ca}_v3.2$ -T-type channels, and chloride channels expressed in somatic and peripheral primary afferent pro-nociceptive neurons (43), TRPV1 channels (55), and TRPA1 receptors (39).

The high affinity of sulfur to react with divalent metallic cations may be the underlying mechanism by which H_2S activates $\text{Ca}_v3.2$ -T-type channels. It has been proposed that H_2S chelates extracellular Zn^{2+} normally bound to the channel, thereby opening the channel (40). Opening of K_{ATP} channels in vascular smooth muscle may be due to intracellular acidification, which in turn is due to activation of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (32).

Recent evidence suggests that the thiol residues of many of the previously mentioned targets are sulfhydrated by H_2S (46). Sulfhydration appears to be a major posttranslational modification of actin, GAPDH, tubulin, and the cysteine residue of K_{ATP} (46). Although sulfhydration may be an attractive explanation of the biologic effects of H_2S , some facts suggest that this mechanism lacks the specificity and reversibility needed to be considered receptor binding. Sulfhydration is a covalent modification of existing thiol residues. Although covalent modification of cysteine residues has been proposed as the activation mechanism of TRPA1 receptors (39), this mechanism is not well characterized and deviates from well-characterized reversible ligand-receptor interactions (26). To terminate receptor activation caused by sulfhydration, desulfhydration through currently unknown mechanisms would have to be part of the process. Ligand binding of numerous neurotransmitter receptors is altered by the redox manipulation of extracellular cysteine residues (for example: 60 and 65) suggesting that H_2S may act on these receptors as well. The potential modification of a wide variety of membrane proteins through sulfhydration would suggest that sulfhydration is a nonselective mechanism of action, which would argue against the existence of specific H_2S receptors activated by sulfhydration. If this mechanism remains the only mechanism of action of H_2S , its promiscuity, in the strict sense, would argue against H_2S qualifying as a gaseous signal molecule.

The action of H_2S in the gastrointestinal tract

H_2S acts on nervous tissue. In the gastrointestinal tract, exogenous application of NaHS has tetrodotoxin-sensitive, prosecretory effects in the guinea pig colon through sensory nerve endings that send collaterals to the mucosa or to secretomotor neurons, which cause secretion, and excites some neurons in the guinea pig myenteric plexus (55). Luminal application of NaHS in the colon increases cFos staining in the spinal cord and enhances nociceptive reflexes, suggesting that H_2S activates primary afferent neurons that innervate the colon (43). This effect, somewhat surprising, given the rapid conversion of luminal H_2S to thiosulfate (34), appears to be mediated by activation of T-type voltage-gated calcium channels (43). In recent experiments done in our laboratory, we found that NaHS (100 μM) *in vitro* facilitates synaptic transmission in mouse sympathetic ganglion neurons that innervate the colon (Fig. 4).

H_2S acts on smooth muscle. With few exceptions, exogenously applied NaHS inhibits gastric and intestinal motility. Circular muscle from guinea pig ileum precontracted with acetylcholine is relaxed by NaHS in a concentration-dependent manner (22). Application of NaHS reduces spontaneous and acetylcholine-induced contractions in the rabbit and rat ileum, effects not blocked by glibenclamide, suggest-

FIG. 4. NaHS-induced facilitation of a fast excitatory postsynaptic potential in a mouse superior ganglion neuron evoked by stimulating the lesser splanchnic nerve. Data are provided courtesy of Dr. Lei Sha.

ing that K_{ATP} channels are not involved (64). NaHS also has an inhibitory effect on muscle contraction in the human, rat, and mouse colon, as well as in the mouse jejunum (17). The inhibitory effect of NaHS in the mouse is unaffected by tetrodotoxin, NOS inhibitors, and PPADS and is retained in the TRPV1-knockout mouse, suggesting that the action of NaHS is directly on smooth muscle cells (17). The direct inhibitory effect is largely through an action on multiple potassium channels, particularly apamin-sensitive small conductance channels and glibenclamide-sensitive K_{ATP} channels (17). In the mouse and human colon, the hyperpolarizing response to NaHS (1 mM) *in vitro* was not blocked by the K_{ATP} channel blocker glibenclamide (20 μM), suggesting that the response to NaHS is not mediated through K_{ATP} channels (Fig. 5).

H_2S acts on ICC. In the gastrointestinal tract, smooth muscle function can be manipulated indirectly through the pacemaker interstitial cells of Cajal (ICCs) that are responsible for phasic fluctuations in smooth muscle membrane potential. Conflicting evidence regards the presence of CBS and CSE in ICCs. ICCs in the guinea pig colon are immunopositive for CSE but not for CBS (55), whereas in preliminary data reported in abstract form, no evidence for CSE and CBS mRNA was found in ICCs from the mouse colon (27). However, an

FIG. 5. Hyperpolarization of mouse circular smooth muscle induced by NaHS (1 mM, applied locally with a micropipette) was not blocked by glibenclamide. Data are provided courtesy of Dr. Lei Sha.

action of H_2S on ICCs remains a possibility, irrespective of whether ICC synthesizes H_2S , because NaHS reduces the frequency of phasic contraction (17). Preliminary data presented in abstract form show that NaHS (100 to 300 μM) depolarizes ICCs and activates the nonselective cation channels responsible for pacemaker activity and slightly enhances spontaneous calcium waves, whereas high concentrations of NaHS (1 mM) activate K_{ATP} channels and increase mitochondrial uptake of calcium, causing inhibition (27). None of the effects were blocked by ODO, the adenylate cyclase inhibitor (SQ22536), or the NOS inhibitor L-NAME.

Summary of the Third and Fourth Criteria. It is clear from studies that use exogenous application of NaHS that a variety of effects in the gastrointestinal system may involve a variety of mechanisms. What is lacking is rigorous pharmacologic examination to determine the nature of the antagonism (26). Competitive antagonism demonstrated by Schild analysis remains the standard method to implicate receptor action of a potential protein. Future studies that implicate a potential receptor should use this proven pharmacologic approach to determine whether the suspected protein is indeed a receptor for H_2S .

The Fifth Criterion: Inactivation of H_2S

General interdisciplinary view of the inactivation of H_2S

Several potential mechanisms exist for the degradation of H_2S in biologic samples. H_2S is oxidized in mitochondria to thiosulfate (4, 34, 72), methylated in the cytosol to methanethiol and dimethylsulfate (69), and sequestered with macromolecules (56). To determine the mechanism by which endogenous H_2S is degraded, it is useful to examine the mo-

lecular fate of $\text{H}_2^{[35]\text{S}}$ applied to tissues. Tracer delivered systemically is recovered as sulfate excreted in the urine (10). When incubated with homogenized tissue samples, or vascularly perfused through the colon, liver, or lung, >90% of the tracer is recovered as either thiosulfate or sulfate, with no evidence of methanethiol production (3, 34). Levitt and colleagues (34) found that within 1 min of delivering $\text{H}_2^{[35]\text{S}}$ to the lumen of the colon in rats, the tracer is recovered as thiosulfate in the portal vein, and as sulfate from blood in the heart (34). They concluded from these studies that tissues oxidize H_2S to thiosulfate, which, after one pass through the liver, is oxidized further to sulfate. Therefore, the likely enzyme that inactivates H_2S is a sulfide oxidase expressed in tissues.

The microbiology of sulfur-oxidizing bacteria provides some clues regarding vertebrate degradation of H_2S through oxidation (14). In early studies of intestinal H_2S gas, Lehmann (33) noticed that H_2S , although found in high concentrations in the proximal colon, was always reduced in the flatus and was never observed in blood, suggesting that a system of H_2S degradation occurs. The first demonstration of biologic sulfide oxidation came from Winogradsky in 1889 (73). Animals from sulfide-rich habitats exhibit aerobic chemotropic electron transport or anaerobic phototropic electron transport through sulfide oxidation (20). Although oxidation of elemental sulfur and thiosulfate occurs in prokaryotic bacteria through the sulfur oxidase (*sox*) family of genes, especially the *soxC/D* gene (15), the major biochemical pathway of H_2S oxidation in eukaryotes is by sulfide quinone reductase (SQR) (20). The crystal structure of bacterial SQR was recently solved by two independent groups (8, 42) (Fig. 6). No transmembrane domains of this protein exist, but rather, it is embedded in the matrix side of the inner membrane of the

FIG. 6. Ribbon diagram of a trimer of *Aquifex aeolicus* sulfide quinone reductase (SQR) derived from x-ray crystallography data. Each monomer is colored in slightly different shades of gray. Flavin adenine dinucleotide (FAD) is shown in black. Reproduced with permission from Marcia and colleagues (42).

mitochondrion. The protein, as a member of the disulfide oxidoreductase flavoprotein (DiSR) superfamily, contains an FAD-binding site that positions FAD adjacent to three cysteine residues that are thought to be the catalytic site of the enzyme. A quinone-binding site is on the *si*-face of FAD, which accepts the electrons given up by H_2S as they create sulfane sulfur that exists briefly as persulfides on SQR cysteine residues. In bacteria, the reaction may stop here, with the elemental sulfur contained in the persulfides being used to generate large polysulfides (8).

Most of our knowledge regarding H_2S degradation in vertebrates comes from the detoxification of H_2S by the colonic mucosa. Because H_2S concentrations in excess of 1,000 ppm have been measured in gas samples obtained from the rat cecum (59), and H_2S can readily cross membrane barriers, a detoxification pathway must exist. Whereas Levitt and colleagues (34) suggested a sulfide oxidase of unknown molecular identity, Picton and colleagues (53) suggested that thiosulfate sulfurtransferase (TST), or rhodanese, was the major enzymatic-degradation pathway. Since its discovery, rhodanese, expressed by the liver, has been known as the enzyme responsible for cyanide detoxification. Cyanide is transferred to thiosulfate to create thiocyanate and sulfite. Picton and colleagues (52) used cyanide in colonic mucosal preparations, showed that thiocyanate is the major product of H_2S degradation, and used this reaction as a surrogate for measuring H_2S consumption to suggest that detoxification is impaired during colitis (52). After reexamination of their data, it appears that TST is involved in H_2S oxidation only when cyanide is present (72) but is likely involved in formation of thiosulfate from oxidized sulfane persulfides (21).

The vertebrate enzyme that exhibits sulfide oxidase activity appears to be SQR (21). Human SQR is encoded by the gene SQR domain-like (SQRL), which is located on chromosome 15 (15q15; Entrez GeneID: 58472). Oxidation from H_2S to sulfane persulfides on SQR occurs as described earlier for bacteria, but instead of the creation of polysulfides, other enzymes are involved to create thiosulfate. The proposed mechanism is that some of the persulfides are oxidized further to sulfite through the action of the enzyme sulfur dioxygenase, which currently does not have a molecular identity that matches its enzymatic activity. The resultant sulfite reacts with persulfides on SQR through the enzymatic action of TST to generate thiosulfate, which is excreted from the cell by an unknown mechanism. It is important to note that in native conditions, no detectable intermediates exist between H_2S and thiosulfate, such that these three enzymes appear to work together so that the complete oxidation of H_2S to thiosulfate occurs instantaneously (21).

Several studies demonstrated that the oxidation of H_2S by SQR is sensitive to the levels of H_2S (20, 21). Concentrations of H_2S in excess of 300 μM reduce the ability of SQR to oxidize H_2S . The biochemistry of this enzyme might underlie the apparently differential effects of slow and fast H_2S -releasing compounds. Quinone-based antibiotics are potent inhibitors of SQR by inhibiting the ability of the enzyme to transfer electrons to quinone (20).

Inactivation of H_2S in the gastrointestinal tract. The mucosa of the gastrointestinal tract, especially the colon, is a model system of H_2S degradation because of its role in detoxifying bacterially derived H_2S and thus has been incorpo-

rated into the previous discussion. The inactivation of endogenously derived H_2S in the gastrointestinal tract remains unknown. Recent experiments in our laboratory show that stigmatellin (3 μM) can inhibit H_2S consumption (Fig. 7). These data implicate SQR in the degradation of H_2S by vertebrate tissues. Because the structure of the catalytic domain of SQR was recently identified (8, 42), it is reasonable to expect that new and more-specific inhibitors of SQR will be forthcoming.

Summary of the Fifth Criterion. Based on isotope-tracing studies, it seems that the major inactivation of H_2S signaling is through oxidation to thiosulfate. The mitochondrial matrix protein SQR is likely the molecular identity of sulfide oxidase responsible for catalyzing this reaction. Therefore, a specific enzymatic-degradation pathway inactivates H_2S , fulfilling the fifth criterion. What remains to be determined is whether pharmacologic manipulation of sulfide oxidation alters endogenous H_2S signaling. The development of new SQR inhibitors as pharmacologic tools will be helpful.

Future Directions

The pharmacologic effects of H_2S generated from NaHS on intracellular metabolic and second-messenger systems, on posttranslational modification of membrane ion channels and transporters and in cell-to-cell communication, are now regarded as established and credible. The lack of solid data and evidence on the function of endogenously generated H_2S gas in the living intact organism is a major impediment to providing an essential link to clinical situations. CBS-knockout mice have hyperhomocysteinemia but lack a demonstrable phenotype that suggests an endogenous role of H_2S produced by CBS (68). Perhaps the absence of an H_2S -related phenotype

FIG. 7. Facsimiles of representative elution peaks of H_2S by using gas chromatography and sulfur fluorescence detection, demonstrating that consumption of H_2S by the external muscle layers of the colon was partially inhibited by the SQR inhibitor stigmatellin. Mouse colonic muscle tissue was incubated in 40 μl normal Krebs' solution with either 3 μM stigmatellin or 0.1% ethanol (vehicle) in 20 ml of 3.6 ppm H_2S gas in 97% O_2 and 3% CO_2 . After 30 min of incubation at 37°C, the gas space was assayed for H_2S .

is due to the compensatory role of 3MST in the brain (23). A recent report of mice lacking CSE demonstrates phenotypic effects on blood pressure, supporting a role for endogenous H₂S in the vascular system (75), but phenotypes in other systems, including the gastrointestinal tract, have not been described. Although considerable data exist in animal models on the pharmacologic effects of H₂S and of sulfide-derived molecules in ischemia/reperfusion, Cardioprotection, and a variety of inflammatory conditions, the link to the clinic and the patient requires further study.

Answers to the following questions are required for H₂S to join the list as one of the body's important regulators of physiologic function:

1. What triggers CBS, CSE, and 3MST to generate H₂S?
2. Can endogenously produced H₂S be monitored in real time with probes in intact and living tissue, thereby providing spatiotemporal information regarding the concentration of H₂S in the environment of the cells?
3. Do receptors for H₂S demonstrate saturable, reversible binding that can be antagonized in a competitive manner?
4. Can stable H₂S-based molecules, rather than H₂S donors, be synthesized so that classic methods to investigate ligand–receptor interactions, such as radioligand binding, be used to define H₂S receptors?
5. Do the catabolic enzymes that convert H₂S to inactive sulfate form an effective barrier and metabolic compartmentalization, greatly limiting local diffusion and intercellular communication?

Looking beyond H₂S to the field of gaseous signaling molecules in general, ammonia and methane may in time enter the stage as gaseous signaling molecules. In addition to the critical role that renal ammonium production and excretion play in acid secretion, and as a principal nitrogen source for microorganisms and plants, it functions as a morphogen during the development of slime molds (78). Just as the active moiety of H₂S appears in some instances to be HS[−], the ammonium ion (NH₄⁺) has important physiologic ramifications. NH₄⁺ transporters have been described in glial cells of the bee retina, where they appear to play an important role in energy metabolism (41). Like HS[−], NH₄⁺ produces a robust intracellular acidification, and NH₄⁺ is endogenously generated and released into the intercellular space of living tissue (9). Methane is potentially another gaseous signaling molecule. Methane slows intestinal transit in the dog and increases the contractile strength of the guinea pig ileum *in vitro* (54). No evidence is available that methane is produced by endogenous tissues, but it is produced by commensal bacteria, and bowel cleansing but not antibiotic treatment reduces exhaled methane (51).

Summary and Conclusions

Several lines of evidence suggest that H₂S is an endogenous gaseous signal molecule in the gastrointestinal tract, but work still remains. Whereas H₂S is enzymatically produced, no evidence indicates that its production is regulated. Although exogenous H₂S exerts several well-defined physiologic effects in the gastrointestinal tract, no study has demonstrated a receptor for H₂S. An enzymatic pathway exists in gastrointestinal tissues for the degradation of H₂S

through oxidation, but no study has demonstrated that manipulation of sulfide oxidation alters H₂S signaling. Future work in the field of gasotransmitters is likely to be demanding. A critical need exists to develop novel technologies, such as H₂S probes and inhibitors of the synthetic and catabolic enzymes. The road ahead for the field remains unclear, but as work in H₂S biology progresses, more surprises and exciting results are expected.

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Abbreviations Used

3MST = 3-mercaptopyruvate sulfurtransferase
cAMP = cyclic adenosine monophosphate
CBS = cystathionine- β -synthase
CNS = central nervous system
CO = carbon monoxide
CSE = cystathione- γ -lyase
EDRF = epithelium-derived relaxing factor
FAD = flavin adenine dinucleotide
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
H₂S = hydrogen sulfide
ICCs = interstitial cells of Cajal
KATP = ATP-sensitive potassium channel
NMDA = *N*-methyl-D-aspartic acid
NO = nitric oxide
ODQ = 1H[1,2,4] oxadiazolo-[4,3- γ] quinoxalin-1-one
PLP = pyroxydyl-5-phosphate
sGC = soluble guanylate cyclase
SQR = sulfide quinone reductase
TRP = transient receptor potential