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# Eukaryotic and prokaryotic contributions to colonic hydrogen sulfide synthesis

**Kyle L. Flannigan,[1] Kathy D. McCoy,[1,2] and John L. Wallace[1]**

[1]*Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada; and* [2]*Maurice E. Müller Laboratories, University Clinic for Visceral Surgery and Medicine, University of Bern, Bern, Switzerland*

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**Flannigan KL, McCoy KD, Wallace JL.** Eukaryotic and prokaryotic contributions to colonic hydrogen sulfide synthesis. *Am J Physiol Gastrointest Liver Physiol* 301: G188–G193, 2011. First published April 7, 2011; doi:10.1152/ajpgi.00105.2011.—Hydrogen sulfide ($H_2S$) is an important modulator of many aspects of digestive function, both in health and disease. Colonic tissue $H_2S$ synthesis increases markedly during injury and inflammation and appears to contribute to resolution. Some of the bacteria residing in the colon can also produce $H_2S$. The extent to which bacterial $H_2S$ synthesis contributes to what is measured as colonic $H_2S$ synthesis is not clear. Using conventional and germ-free mice, we have delineated the eukaryotic vs. prokaryotic contributions to colonic $H_2S$ synthesis, both in healthy and colitic mice. Colonic tissue $H_2S$ production is entirely dependent on the presence of the cofactor pyridoxal 5′-phosphate (vitamin $B_6$), while bacterial $H_2S$ synthesis appears to occur independent of this cofactor. As expected, approximately one-half of the $H_2S$ produced by feces is derived from eukaryotic cells. While colonic $H_2S$ synthesis is markedly increased when the tissue is inflamed, and, in proportion to the extent of inflammation, fecal $H_2S$ synthesis does not change and tissue granulocytes do not appear to be the source of the elevated $H_2S$ production. Rats fed a B vitamin-deficient diet for 6 wk exhibited significantly diminished colonic $H_2S$ synthesis, but fecal $H_2S$ synthesis was not different from that of rats on the control diet. Our results demonstrate that $H_2S$ production by colonic bacteria does not contribute significantly to what is measured as colonic tissue $H_2S$ production, using the acetate trapping assay system employed in this study.

inflammation; mucosal defense; ulcer; repair; bacteria; microflora

WHILE OFTEN REGARDED AS A toxic gas (12), hydrogen sulfide ($H_2S$), like nitric oxide and carbon monoxide, is increasingly recognized as an endogenous gaseous mediator with many physiological functions in the body (19, 40). $H_2S$ may also have considerable therapeutic potential (11, 29, 31, 38). $H_2S$ is produced in most mammalian tissues and contributes to fundamental processes such as vasodilation (42), neuromodulation (1, 15), leukocyte-endothelial adhesion (41), smooth muscle relaxation (14), and nociception (7, 8). In the gastrointestinal tract, $H_2S$ has been shown to enhance ulcer healing (35), to promote the resolution of colitis (37), and to contribute to gastric mucosal defense (9, 10, 39).

$H_2S$ is produced in mammalian tissue from the amino acid cysteine. Catabolism of cysteine into $H_2S$, pyruvate, and ammonia occurs via two pyridoxal 5′-phosphate (P-5-*P*)-dependent enzymes: cystathionine-γ-lyase and cystathionine-β-synthase (19, 31, 40). The synthesis of $H_2S$ throughout the gastrointestinal tract of mice and rats, and of human colon biopsies, has been characterized using the zinc acetate trapping method (22). This same technique was employed to measure $H_2S$ production in the inflamed rat colon (37). $H_2S$ was found to be increased >100-fold within 24 h of induction of colitis via administration of trinitrobenzene sulfonic acid (TNBS). Inhibition of $H_2S$ synthesis resulted in impaired resolution of the inflammation, while administration of $H_2S$ donors accelerated repair (37).

Bacteria residing in the lumen of the gastrointestinal tract can also produce $H_2S$, and recent studies suggest that colonocytes and other cells can use it as an energy source (13, 16). The concentration of $H_2S$ in the lumen of human and mouse colon has been reported by some investigators to be in the millimolar range (2, 26). However, others have provided evidence that most of the sulfide content in the colonic lumen exists in a bound form in fecal material (17, 25), so levels of "free" $H_2S$ are more likely to be in the micromolar range (17). The extent to which $H_2S$ produced by luminal bacteria can gain access to the colonic mucosa is not clear. While $H_2S$ that is not bound to fecal material can freely diffuse across the apical membrane of a colonocyte (23), virtually all absorbed $H_2S$ is oxidized to thiosulfate (13, 18). This oxidation primarily occurs in colonocytes (13), keeping $H_2S$ concentrations at nontoxic levels (16).

One concern with measurements of colonic $H_2S$ synthesis is the potential for contamination of tissue samples by bacteria. Thus a portion of what is measured as colonic $H_2S$ synthesis could in fact be bacterial $H_2S$ synthesis. The present study was undertaken to determine the extent to which $H_2S$ produced by colonic bacteria might contribute to what is measured as "colonic" $H_2S$ synthesis using the widely employed zinc acetate trapping assay (30). To do this, we used conventional and germ-free mice and examined colonic $H_2S$ synthesis in physiological and pathophysiological conditions. The dependence of tissue vs. fecal $H_2S$ synthesis on the presence of P-5-*P* and L-cysteine in the assay was also examined.

## MATERIALS AND METHODS

*Animals.* Male Swiss Webster, NIH Swiss, and NMRI mice and male Wistar rats were housed in plastic cages and maintained under controlled temperature (20°C), humidity (60–70%), and light cycle (12:12-h light-dark). The animals were fed standard laboratory chow and water ad libitum. Germ-free mice of the same strains were derived and maintained germ-free as previously described (27, 28) at the McMaster Axenic Gnotobiotic Unit. Swiss Webster, NIH Swiss, and NMRI mice with altered Schaedler flora (ASF; Taconic) (6) were used as colonized counterparts to germ-free mice. Mice colonized with ASF were kept in individually ventilated microisolator cages in the McMaster Axenic Gnotobiotic Unit. All experimental protocols were approved by the Animal Research Ethics Board at McMaster University and adhered to the guidelines established by the Canadian Council on Animal Care.

*Experimental colitis.* Colitis was induced in conventionally housed mice using the hapten 2,4,6-trinitrobenzene sulfonic acid (TNBS) (24). A mild colitis was induced by intrarectal administration of 100

Address for reprint requests and other correspondence: J. L. Wallace, Farncombe Institute, McMaster Univ., 1280 Main St. West, HSC-3N8, Hamilton, Ontario, L8S4K1, Canada (e-mail: jwalla@mcmaster.ca).

Table 1. *Criteria for scoring colonic damage and inflammation*

| Score | Appearance |
|---|---|
| 0 | Normal |
| 1 | Localized hyperemia, no ulcers |
| 2 | Ulceration without hyperemia or bowel wall thickening |
| 3 | Ulceration with inflammation at one site |
| 4 | Two or more sites of ulceration and inflammation |
| 5 | Ulceration at multiple sites or extending >1 cm along the length of the colon |
| 6–10 | When an area of damage extended >2 cm along the length of colon, the score was increased by 1 for each additional cm of involvement |

In addition, the score was increased by 1 or 2 if there were mild or severe adhesions, respectively, by 1 if diarrhea was evident, and by 1 if rectal bleeding was evident. The maximum colon thickness (mm) was also added to the score.

μl of a 10-mg/ml solution of TNBS in 40% ethanol. A more severe colitis was induced by administering 100 μl of a 20-mg/ml solution of TNBS in 40% ethanol. TNBS administration was performed after lightly sedating the mice with isoflurane. A 5FG pediatric feeding tube catheter was inserted 2.5 cm in the colon. The TNBS was administered slowly (~5 s) while applying pressure around the rectum to prevent leakage. Pressure was maintained while removing the catheter, and the rectum was held closed for 4 min after TNBS administration. Mice were killed by an overdose of isoflurane 3 days later for evaluation of the severity of damage and tissue collection.

Macroscopic colonic damage was assessed using a slightly modified version of that described previously (36), as outlined in Table 1. Damage was scored blindly. Colon thickness was measured using digital calipers. Colonic inflammation was further assessed by the measurement of myeloperoxidase (MPO) activity, as described previously (5). MPO has been used extensively as a quantitative index of granulocyte infiltration (4, 5, 36).

Samples of the distal colon were excised and snap-frozen for subsequent measurement of MPO activity and $H_2S$ synthesis. Samples were taken from the liver for measurement of $H_2S$ synthesis. The samples were taken from areas of ulceration or inflammation making sure to incorporate a margin of macroscopically normal tissue. At the time of death, fecal material was dissected out of the colon. All tissue was snap-frozen in liquid nitrogen immediately after collection and stored at −80°C.

*$H_2S$ synthesis.* The capacity for samples of colon, liver, and feces to produce $H_2S$ was measured using a slightly modified version (37) of a previously described assay (30). P-5-*P* (2 mM) and L-cysteine (10 mM) were generally included in the reagent mixture. However, in some experiments, P-5-*P*, L-cysteine, or both were excluded from the assay. A standard curve was generated using various concentrations of NaHS.

*Effects of vitamin B deficiency.* Rats were provided one of two diets for 6 wk. One diet lacked vitamin $B_6$, vitamin $B_{12}$, and folate and has previously been shown to induce hyperhomocysteinemia (33). Because the major enzymes for $H_2S$ synthesis require vitamin $B_6$, this diet would be expected to cause an impairment of $H_2S$ synthesis. The control diet was identical but contained the above-mentioned vitamins and folate. Both diets were prepared by Harlan Teklad (Madison, WI) and contained 1% sulfathiozole (10 g/kg diet) to inhibit folate formation by gut bacteria (33).

*Isolation of rat peritoneal neutrophils.* Rats were lightly sedated with isoflurane, and 20 ml of a 1% solution of glycogen (from oyster, type II; Sigma-Aldrich) was injected intraperitoneally (20-G needle). Four hours later the rats were killed by overdose of isoflurane, and a small incision was cut in the abdomen of the rat to allow access to the peritoneum. Heparinized saline (20 ml; 25 U/ml) was injected through the incision, and the abdomen of the animal was manipulated gently to allow the mixing of peritoneal contents. Peritoneal fluid was collected using a pediatric 5FG feeding tube. The peritoneal fluid was centrifuged (300 rpm, 15 min), and the resulting pellet was resuspended in 10 ml of heparinized saline (25 U/ml). Further dilutions of these cells were made in isotonic saline. These various concentrations of cells were assayed for MPO activity and $H_2S$ synthesis following the same procedures as described above.

*Materials.* L-Cysteine, P-5-*P*, $FeCl_3$, *N*,*N*′-dimethyl-*p*-phenylenediamine sulfate salt, sulfathiozole, zinc acetate, TNBS, and NaHS were obtained from Sigma-Aldrich (St. Louis, MO). Isoflurane was obtained from Abbott Laboratories (Montreal, Canada).

*Statistical analysis.* All data are expressed as means ± SE. Linear regression analyses were performed using GraphPad Prism version 5.0. Comparisons of data were performed using a one-way ANOVA and the Dunnett's multiple-comparison test. An associated probability (*P* value) of <5% was considered significant.

## RESULTS

*Colonic $H_2S$ synthesis is similar in germ-free and colonized mice.* As shown in Fig. 1, colonic $H_2S$ synthesis was similar in Swiss Webster mice that were germ-free and those colonized with ASF. Similar findings were obtained with two other strains of mice (NMRI and NIH), but the sample sizes in the germ-free groups were small (3 and 2, respectively). There was also no significant difference in liver $H_2S$ synthesis between germ-free and ASF-colonized mice (1,172 ± 207 vs. 826 ± 143 $nmol \cdot g^{-1} \cdot h^{-1}$, respectively).

*Eukaryotic and prokaryotic contributions to fecal $H_2S$ synthesis.* Both colonic tissue $H_2S$ synthesis and fecal $H_2S$ synthesis are dependent on the presence of L-cysteine in the reaction mixture (Fig. 2). However, while colonic tissue $H_2S$ synthesis is completely P-5-*P*-dependent, fecal $H_2S$ production is only ~50% dependent on the presence of P-5-*P*. This is likely a reflection of bacterial cells comprising only ~50% of fecal mass (32). Thus the P-5-*P*-independent portion of fecal $H_2S$ synthesis likely represents the prokaryotic contribution. This conclusion is supported by the observation that fecal $H_2S$ synthesis in germ-free mice is only ~50% of that observed in colonized mice (Fig. 2). It is noteworthy that a portion of fecal $H_2S$ synthesis in germ-free mice was not dependent on the presence of P-5-*P* in the reaction mixture.

*Colonic damage/inflammation affects colonic but not fecal $H_2S$ synthesis.* Induction of colitis resulted in increases in colonic $H_2S$ synthesis, with the greatest increases seen in animals with the most severe colitis (Fig. 3, *A*–*C*). Indeed, colonic $H_2S$ synthesis correlated very well with tissue MPO

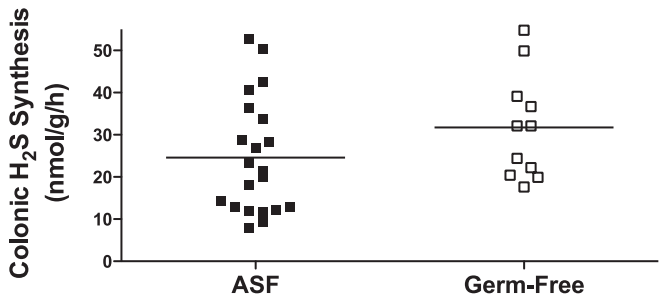


Fig. 1. Colonic hydrogen sulfide ($H_2S$) synthesis did not differ significantly between germ-free mice and the same strains of mice colonized with altered Schaedler flora (ASF). Both groups include Swiss Webster, NIH Swiss, and NMRI mice.

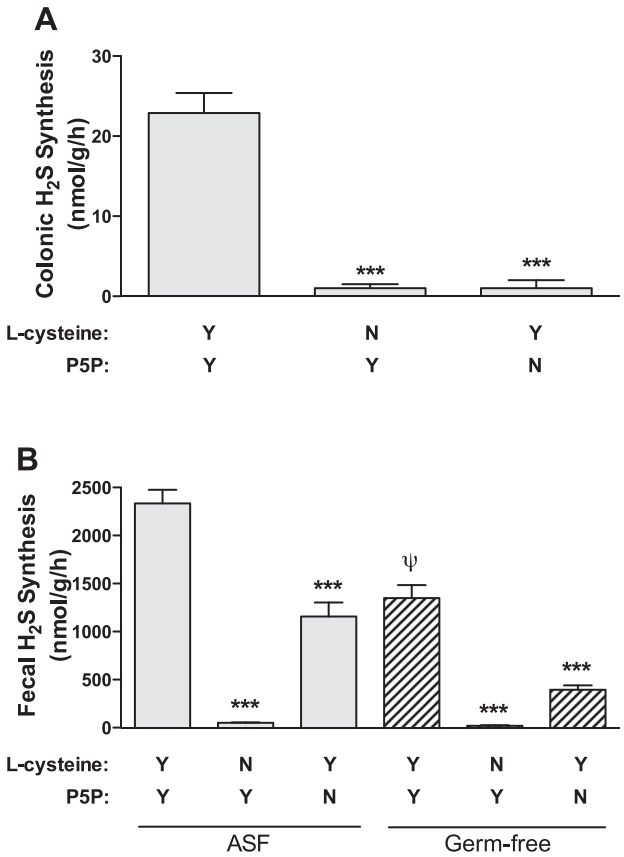


Fig. 2. Requirement for L-cysteine and pyridoxal 5′-phosphate [P-5-*P* (P5P)] in $H_2S$ synthesis by mouse colonic tissue and feces. The presence of L-cysteine or P-5-*P* is indicated by "Y" and the absence by "N." *A*: colonic $H_2S$ synthesis in NIH Swiss mice. $H_2S$ synthesis is almost completely dependent on the presence of both L-cysteine and P-5-*P*. *B*: fecal $H_2S$ synthesis in NIH Swiss mice that were raised germ-free compared with NIH mice colonized with ASF. While completely dependent on the presence of L-cysteine, fecal $H_2S$ synthesis in ASF and germ-free mice is only ~50% and ~70% dependent on P-5-*P*, respectively. Note: colonic $H_2S$ synthesis did not differ significantly between conventionally housed and ASF-colonized NIH Swiss mice (data not shown). $^{***}P < 0.001$ vs. the corresponding group with both L-cysteine and P-5-*P*. $^{\Psi}P < 0.05$ vs. the corresponding ASF group.

activity (a marker of granulocyte infiltration) (Fig. 3*E*). In contrast, fecal $H_2S$ synthesis was not altered when the colon was inflamed, compared with healthy controls, and there was no such correlation between tissue MPO activity and fecal $H_2S$ synthesis (Fig. 3, *D* and *F*). Given the correlation between tissue MPO activity and tissue $H_2S$ synthesis, it was possible that neutrophils were a significant source of the measured $H_2S$. To explore this, we measured $H_2S$ synthesis by neutrophils harvested from the peritoneum (following ip injection of oyster glycogen). As shown in Fig. 4, there was no correlation between the concentration of neutrophils and the amount of $H_2S$ produced (although the concentration of neutrophils did correlate very well with MPO activity; $R^2 = 0.98$; $P > 0.001$).

*Vitamin B deficiency reduces colonic but not fecal $H_2S$ synthesis.* As reported previously (33), the rats fed a vitamin B-deficient diet for 6 wk developed significant hyperhomocysteinemia (plasma homocysteine levels of 189 ± 24 vs. 10 ± 2 μmol/l in rats fed the control diet). This was accompanied by an almost complete (~95%) reduction of colonic tissue $H_2S$ synthesis compared with controls ($P < 0.05$; Fig. 5*A*). However, fecal $H_2S$ synthesis did not differ significantly between the two diets at the beginning or at the end of the 6-wk period (Fig. 5*B*).

## DISCUSSION

$H_2S$ affects many aspects of digestive function, including blood flow, visceral sensitivity, smooth muscle contraction, and secretion (19, 40). It also appears to play an important role in regulating inflammatory processes in the gastrointestinal tract. Thus suppression of $H_2S$ synthesis results in a significant increase in granulocyte numbers in the mucosa, downregulation of cyclooxygenase-2 expression, and reduced prostaglandin $E_2$ synthesis (37). On the other hand, $H_2S$ donors can accelerate resolution of experimental colitis (37), protect the gastric mucosa from injury induced by nonsteroidal anti-inflammatory drugs (9, 34, 38, 39), and accelerate the healing of gastric ulcers (34, 35). Previous studies have characterized $H_2S$ production throughout the gastrointestinal tract (22), demonstrating that the amounts produced on a per gram basis are somewhat greater in the stomach and small intestine than in the colon. Previous studies have also shown (35, 37), as in the present study, that colonic $H_2S$ synthesis is markedly increased when the tissue is inflamed/damaged. In the present study, we observed a significant correlation between tissue MPO activity (a marker of granulocyte infiltration) and tissue $H_2S$ synthesis. However, studies of isolated neutrophils failed to show any such correlation, suggesting that neutrophils were unlikely to be the cellular source of the elevated $H_2S$ synthesis in the inflamed colon.

Of course, in addition to being produced by gastrointestinal tissue, many species of colonic bacteria can also produce $H_2S$. While colonocytes may act as a "metabolic barrier" to diffusion of luminally derived $H_2S$ in the subepithelial compartment (by efficiently catabolizing $H_2S$) (13, 16), it remains possible that some $H_2S$ produced by bacteria could significantly affect colonic function. Moreover, it has been suggested that sulfate-reducing bacteria within the lumen of the intestine could contribute to what is measured as tissue production of $H_2S$ (20). This possibility was examined in the present study, and our results strongly suggest that there is no significant contribution of colonic bacteria to the $H_2S$ synthesis measured in samples of colonic tissue. The main evidence for this conclusion is the following: *1*) colonic $H_2S$ synthesis did not differ significantly between germ-free and colonized mice; *2*) $H_2S$ production by inflamed colonic tissue increased proportionately with the severity of inflammation (as measured by MPO activity), but fecal $H_2S$ production remained unchanged; and *3*) feeding rats a vitamin B-deficient diet for 6 wk almost completely abolished colonic $H_2S$ synthesis but had no effect on fecal $H_2S$ synthesis. Interestingly, rats on the vitamin B-deficient diet displayed significantly reduced fecal $H_2S$ synthesis after 2 wk on the diet, which is consistent with a portion of fecal $H_2S$ synthesis (i.e., the eukaryotic portion) being vitamin $B_6$ (P-5-*P*) dependent. The observation that, by the end of 6 wk on the vitamin B-deficient diet, the fecal $H_2S$ production had returned to the same levels as in controls may suggest changes in the microflora of these rats or in the production of $H_2S$ via alternative pathways by colonic bacteria.

*Escherichia coli*, *Salmonella enterica*, *Clostridia*, and *Enterobacter aerogenes*, all of which can be found in the large

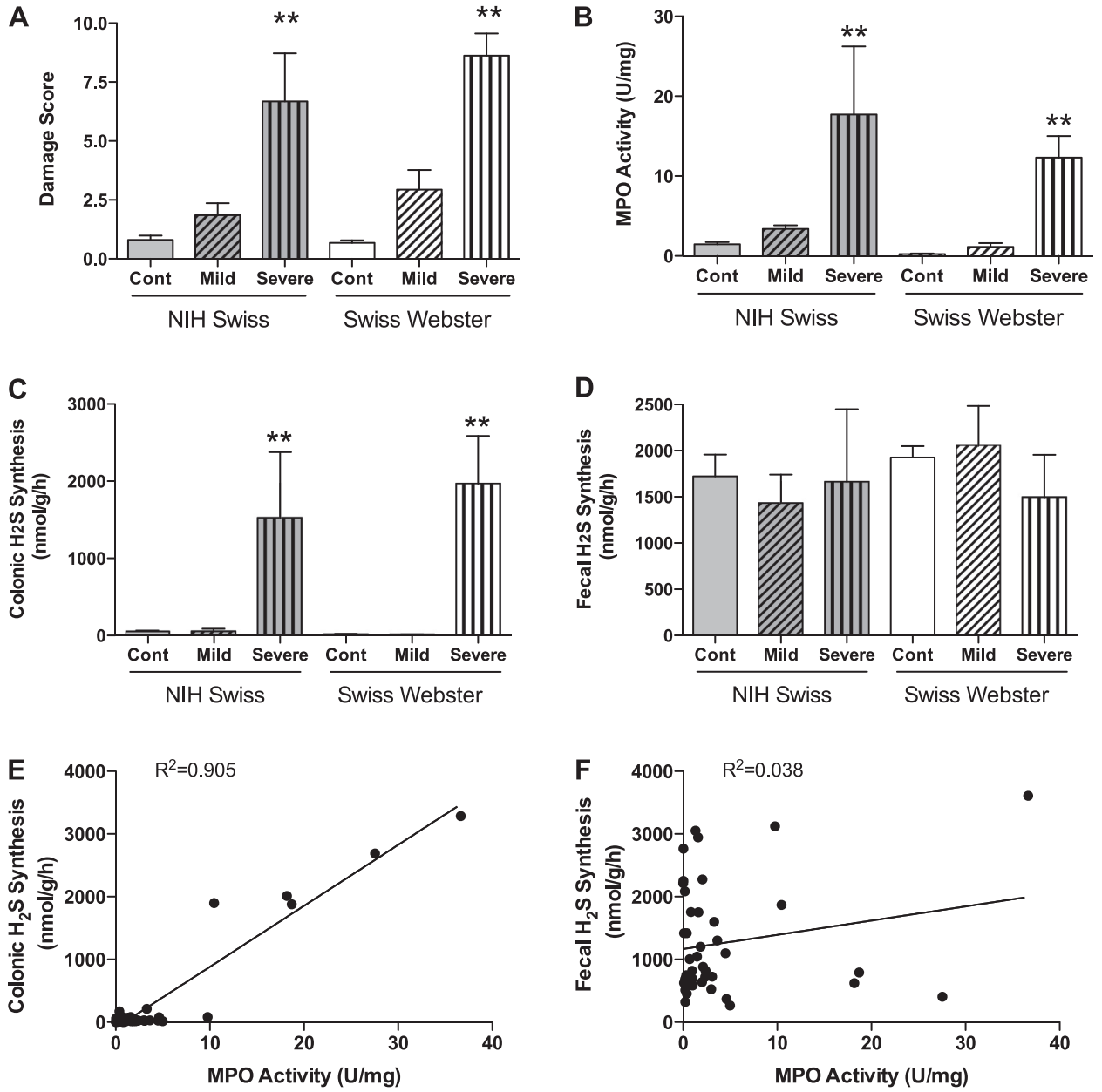


Fig. 3. Differences in colonic vs. fecal $H_2S$ synthesis in healthy mice or mice with mild or severe colitis (induced by trinitrobenzene sulfonic acid). Colonic $H_2S$ synthesis is markedly elevated when the colon is inflamed (*A–C*), whereas fecal $H_2S$ synthesis remains unchanged (*D*). Colonic (*E*), but not fecal (*F*), $H_2S$ synthesis correlates very well with tissue inflammation [as measured by myeloperoxidase (MPO) activity, a marker of granulocyte infiltration]. $^{**}P < 0.01$ vs. the corresponding control (Cont) group.

intestine, are among the bacteria that can metabolize cysteine to $H_2S$ (3). $H_2S$ is also produced in the colon by sulfate-reducing bacteria that have the ability to reduce sulfate and other sulfur-containing compounds to $H_2S$ (3). Based on studies in which P-5-*P* was excluded from the reaction mixture in the $H_2S$ assay, it would appear that fecal $H_2S$ synthesis is derived ~50% from bacteria and ~50% from eukaryotic cells. This is consistent with bacterial cells comprising about one-half of fecal mass (32). Colonic (eukaryotic) $H_2S$ synthesis is almost entirely dependent on P-5-*P*. Fecal $H_2S$ synthesis was reduced by 50% in the absence of P-5-*P*, likely corresponding to inhibition of the eukaryotic component. The observation that fecal samples from germ-free mice produced only about one-half as much $H_2S$ as fecal samples from colonized mice supports this conclusion.

$H_2S$ is a highly reactive gaseous mediator that can freely diffuse across membranes (23). It is rapidly metabolized by the mitochondrial enzyme sulfide quinone reductase (16) and can be used as an alternative to oxygen in mitochondrial respiration (13, 16). Colonocytes appear to be particularly well adapted to using $H_2S$ as a metabolic fuel (13). $H_2S$ can also be sequestered, such as through binding to heme proteins (19). Thus, measurement of tissue or plasma levels of $H_2S$ in vivo has proven to be very challenging. Unlike the case for nitric oxide, another gaseous mediator, no suitable biomarkers for $H_2S$ have been identified (as nitrate/nitrite

are for nitric oxide) (19). This presents significant challenges in terms of identification of physiological and pathophysiological roles for this gas (19).

The most commonly employed assay for measurement of tissue production of $H_2S$ is the zinc acetate trapping technique first described by Stipanuk and Beck (30). This assay has been used for determination of $H_2S$ synthesis in liver and kidney, gastrointestinal tissues, brain, serum, vascular tissues, and pancreas (see Ref. 19 for a recent review). The assay may be viewed as a measure of the capacity of a tissue to produce $H_2S$, since, in most cases, very little $H_2S$ can be detected in the absence of added substrate (L-cysteine). Linden et al. (21) recently described an elegant method for measuring tissue $H_2S$ production in vitro in the absence of exogenous substrate.

In recent years, there has been considerable evidence emerging for important roles of $H_2S$ in many aspects of digestive health and disease. Tissue production of $H_2S$ appears to be important for maintenance of mucosal integrity, for modulation of inflammation, and for promotion of tissue repair (9, 35, 37, 39). The fact that $H_2S$ can be used as a metabolic fuel, particularly by colonocytes, further underscores its potential importance in health and disease. The extent to which bacteria-derived $H_2S$ can modulate mucosal function remains unclear, given the ability of the epithelium to rapidly catabolize this gaseous mediator. On the other hand, in circumstances of epithelial damage or dysfunction, it is possible that the metabolic barrier to diffusion of $H_2S$ in the subepithelial compartment is impaired. The present study provides evidence that, using the most widely applied assay for tissue $H_2S$ synthesis, bacteria do not make a significant contribution to what is measured as colonic $H_2S$. Colonic $H_2S$ synthesis is markedly upregulated when the tissue is damaged/inflamed and appears to contribute significantly to resolution and repair.

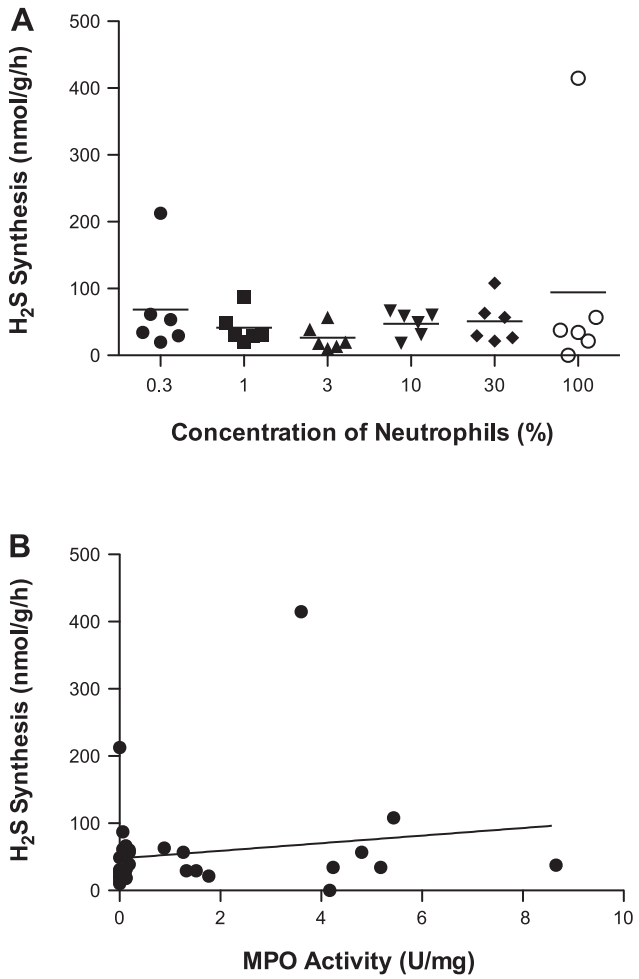


Fig. 4. Production of $H_2S$ by various concentrations of neutrophils harvested from the peritoneal cavity of rats ($n = 6$). There is no correlation between the concentration of neutrophils (*A*) or the MPO activity (*B*; $R^2 = 0.03$; not significant) and the amount of $H_2S$ produced. There was a highly significant linear correlation between the concentration of neutrophils and MPO activity ($R^2 = 0.98$; $P < 0.001$).

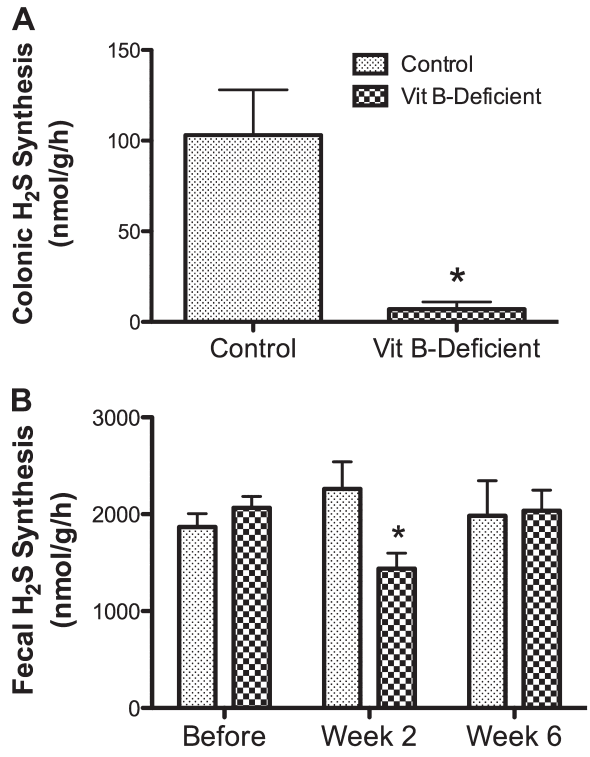


Fig. 5. Consumption by rats of a diet deficient in B vitamins results in almost complete suppression of colonic $H_2S$ synthesis but only a small and transitory effect on fecal $H_2S$ synthesis. *A*: colonic $H_2S$ synthesis after the rats had been on the vitamin B (Vit B)-deficient or control diet. $P < 0.05$ vs. the control group. *B*: fecal $H_2S$ synthesis before and after 2 and 6 wk of consumption of the vitamin B-deficient or control diets. Fecal $H_2S$ synthesis was significantly ($P < 0.05$) decreased after 2 wk on the vitamin B-deficient diet but had recovered to control levels by 6 wk on the vitamin B-deficient diet. $n = 6$ mice/group.


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## GRANTS

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## DISCLOSURES

No conflicts of interest are declared by the authors.

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