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Abstract
Bovine rumen fluid was fermented anaerobically with 25 mM *R*-propylene glycol, *S*-propylene glycol, or glycerol added. After 24 h, all of the propylene glycol enantiomers and approximately 80% of the glycerol were metabolized. Acetate, propionate, butyrate, valerate, and caproate concentrations, in decreasing order, all increased with incubation time. Addition of any of the three substrates somewhat decreased acetate formation, while addition of either propylene glycol increased propionate formation but decreased that of butyrate. *R*- and *S*-propylene glycol did not differ significantly in either their rates of disappearance or the products formed when they were added to the fermentation medium. Fermentations of rumen fluid containing propylene glycol emitted the sulfur-containing gases 1-propanethiol, 1-(methylthio)propane, methylthiirane, 2,4-dimethylthiophene, 1-(methylthio)-1-propanethiol, dipropyl disulfide, 1-(propylthio)-1-propanethiol, dipropyl trisulfide, 3,5-diethyl-1,2,4-trithiolane, 2-ethyl-1,3-dithiane, and 2,4,6-triethyl-1,3,5-trithiane. Metabolic pathways that yield each of these gases are proposed. The sulfur-containing gases produced during propylene glycol fermentation in the rumen may contribute to the toxic effects seen in cattle when high doses are administered for therapeutic purposes.

Keywords
Biodiesel, glycerol, ketosis, propylene glycol, rumen, sulfur-containing gases, volatile fatty acids

Comments
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Ruminal Fermentation of Propylene Glycol and Glycerol

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Bovine rumen fluid was fermented anaerobically with 25 mM *R*-propylene glycol, *S*-propylene glycol, or glycerol added. After 24 h, all of the propylene glycol enantiomers and approximately 80% of the glycerol were metabolized. Acetate, propionate, butyrate, valerate, and caproate concentrations, in decreasing order, all increased with incubation time. Addition of any of the three substrates somewhat decreased acetate formation, while addition of either propylene glycol increased propionate formation but decreased that of butyrate. *R*- and *S*-propylene glycol did not differ significantly in either their rates of disappearance or the products formed when they were added to the fermentation medium. Fermentations of rumen fluid containing propylene glycol emitted the sulfur-containing gases 1-propanethiol, 1-(methylthio)propane, methylthiirane, 2,4-dimethylthiophene, 1-(methylthio)-1-propanethiol, dipropyl disulfide, 1-(propylthio)-1-propanethiol, dipropyl trisulfide, 3,5-diethyl-1,2,4-trithiolane, 2-ethyl-1,3-dithiane, and 2,4,6-triethyl-1,3,5-trithiane. Metabolic pathways that yield each of these gases are proposed. The sulfur-containing gases produced during propylene glycol fermentation in the rumen may contribute to the toxic effects seen in cattle when high doses are administered for therapeutic purposes.

KEYWORDS: Biodiesel; glycerol; ketosis; propylene glycol; rumen; sulfur-containing gases; volatile fatty acids

INTRODUCTION

Governmental encouragement of a biofuels economy will strongly impact not only crop production agriculture but also animal production agriculture. The projected large-scale conversion of triglycerides to esterified fatty acids for biodiesel will result in glycerol (1,2,3-propanetriol) production greatly exceeding demand (*1*, *2*). Glycerol is now produced by triglyceride hydrolysis, with most of it used for food and personal care products. Although glycerol can be converted to a number of useful compounds, such as ethylene glycol (1,2-ethanediol) and propylene glycol (1,2-propanediol) (*3*, *4*), much of its excess production may go to ruminant feed.

The feeding of sugars and other polyols to ruminants for either therapeutic or nutritional purposes is a long-standing practice in the animal production industry. Ketosis, a metabolic disorder affecting dairy cows that occurs during high-energy demand (milk synthesis) and insufficient nutrient intake (late prepartum to early postpartum), is effectively treated with drenching of propylene glycol (*5*−*8*) and glycerol (*9*, *10*). A diet can be supplemented with proplyene glycol to prevent ketosis; however, feeding supplementary glycerol is not effective in preventing it (*9*, *10*).

Although propylene glycol can prevent and treat ketosis in dairy cows, large doses (>500 g/day) can harm cattle (*5*, *11*) and other herbivores. Symptoms when such doses are fed include depression, ataxia, and excessive salivation, as well as abnormal, malodorous, and foul breath and feces (*12*−*14*). Anecdotal evidence suggests that the foul breath is sulfurous, and specifically garlicky and/or onion-like in odor. As large amounts of glycerol may be fed to ruminants and as propylene glycol may be toxic to them, a greater understanding of how these compounds are metabolized in ruminants is needed.

There are two ways to investigate propylene glycol and glycerol metabolism in ruminants: (1) by *in vivo* experiments with these compounds added either orally or by infusion through a cannula and (2) by *in vitro* experiments with rumen fluid extracted from cannulated animals to which these compounds are then added.

In an *in vivo* experiment, propylene glycol was added to the normal rations of cannulated lactating dairy cows (*15*). Blood plasma concentrations of acetate and glucose decreased, while that of propionate increased. However, propylene glycol infused into emptied and washed rumens of cannulated lactating dairy

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cows resulted in increased blood plasma concentrations of both lactate and propylene glycol throughout the infusion period (*16*). This supports the idea that propylene glycol can be absorbed intact by cows; however, the authors suggest that a majority of propylene glycol metabolism in cattle is due to rumen microbiota. Glycerol fed to the rumens of cannulated dry dairy cows quickly disappeared, and in general it is primarily fermented in the rumen rather than being absorbed through the rumen wall or being removed by flow to the omasum (*17*). When glycerol is dosed into the rumens of cannulated bulls, the ratio of propionate to acetate is increased, although in some cases higher plasma glycerol concentrations were observed, depending upon the nutrient status of the animal (*18*).

Although diffusion of substrate through the rumen wall and bulk flow through the rumen do not occur with *in vitro* experiments, such experiments allow samples to be taken over extensive time intervals, improving researchers' ability to control experimental conditions (*19*). This yields important inferences about substrate uptake and product formation not easily obtained with *in vivo* systems.

We earlier carried out *in vitro* anaerobic fermentation of rumen fluid with cottonseed, alfalfa, or corn added to it (*20*) under much the same conditions as those used in the work described in this article. The work presented here follows that previously described.

The purpose of this paper is to describe the fate of propylene glycol and glycerol when added to rumen fluid from cannulated cattle. This was studied by measuring the uptake of the substrates and the production of acetic, propionic, butyric, valeric, caproic, and lactic acids. Since fermented samples with added propylene glycol had distinctive sulfurous odors, we subjected them to headspace GC−mass spectroscopy−olfactometry analysis (GC−MS−O) to identify the compounds generating these odors and to relate these compounds to the reported toxicity of propylene glycol.

MATERIALS AND METHODS

Rumen Fluid Sampling. Rumen fluid samples were taken from cannulated dairy cows 2 h after they received half of the daily ration. This half-ration comprised 1.8 kg of alfalfa hay, 0.7 kg of sliced grass, 5.2 kg of corn silage, 2.3 kg of cracked corn, 0.9 kg of SoyPLUS, 0.7 kg of soybean meal, 0.5 kg of beet pulp, 0.35 kg of cane molasses, 35 g of $CaCO_3$, 35 g of $Ca_3(PO_4)_2$, 30 g of MgO, 15 g of trace mineral salt mixture, 2 g of trace mineral beef mixture, 25 g of vitamin ADE mixture, and 3 g of vitamin E. Cannulated cows used in this study were approved for research use under an animal care and use protocol at the National Animal Disease Center.

Fermentation with Added Propylene Glycol or Glycerol for Liquid Sampling. The rumen fluid sample was divided into eight 25-mL portions and added to flasks already containing 25 mL of anaerobic dilution buffer, pH 6.8 (*20*). A stock solution of the latter consisted of 75 mL of 0.6% K_2HPO_4, 75 mL of salt solution, 1 mL of 0.1% resazurin, and 800 mL of water, boiled and flushed under a CO_2 atmosphere with 50 mL of 8% Na_2CO_3 added. The salt solution was composed of 0.6% KH_2PO_4, 1.2% $(NH_4)_2SO_4$, 1.2% NaCl, 0.12% $MgSO_4 \cdot 7H_2O$, and 0.12% $CaCl_2 \cdot 6H_2O$. After autoclaving, 20 mL of 2.5% cysteine was added as a reducing agent to the buffer solution. Three pairs of flasks flushed with CO_2 received 25 mM *R*-propylene glycol, *S*-propylene glycol, or glycerol, with the fourth pair having only buffer. Flasks were closed with butyl rubber stoppers vented with 20-gauge needles and were incubated at 37 °C with periodic mixing. Samples of 5 mL were taken at 0, 2, 4, 6, 8, and 24 h and frozen until analysis.

Sample Centrifugation and Filtration. Incubation samples were rapidly thawed and thoroughly mixed. Portions of 2 mL were withdrawn using a pipet with a disposable tip widened with a razor for better access to a representative volume. Equal amounts were centrifuged in 1.5-mL vials at 10000*g* for 10 min. Each sample was filtered with a disposable 0.22-μm cellulose acetate syringe-tip filter.

Sample Derivatization for GC. Two derivatization methods were used to prepare the samples for GC. Butylation (*21*) was used to determine fatty acid concentrations, while trimethylsilylation (TMS) (*22*) was used to determine concentrations of *R*- and *S*-propylene glycol, glycerol, and lactic acid.

To form *n*-butyl esters of the fatty acids in the samples, 500 μL of each sample was added to a 4-mL glass screw-top vial with 100 μL of 8% (w/v) NaOH, and the vial was sealed with an open-top Teflon septum-covered cap. Some samples were then frozen until derivatization. Vials containing the samples were thawed (if they had been frozen), and 300 μL of butylation fluid, consisting of 80% (v/v) *n*-butanol and 20% (v/v) H_2SO_4, was added to each. This was followed by 750 μL of chloroform and 50 μL of a 7.89 g L^{-1} heptanoic acid in chloroform solution, the latter serving as an internal standard. Solutions in capped vials were heated for 2 h at 80 °C and allowed to cool to room temperature before 300 μL of trifluoroacetic acid was added to convert the remaining *n*-butanol to *n*-butyl trifluoroacetate. After 1 h, the mixtures were extracted with three 1-mL portions of deionized water, the aqueous layers being removed with a 1-mL disposable syringe after each extraction. After the third extraction, the desalted organic layers were transferred to clean 2-mL screw-top vials, and the vials were sealed with open-top septum-covered caps.

To produce TMS derivatives of *R*- and *S*-propylene glycol, glycerol, and lactic acid, 300-μL portions of the samples were placed onto 5-cm-diameter glass plates and dried in an oven for 1 h at 45 °C. The concentrated material on each plate was diluted with 200 μL of methanol and transferred into a 1.5-mL microcentrifuge vial. A further 300 μL of methanol was added to each plate and mixed for 5 s before being added to the vial. A portion of 100 μL was added to 750 μL of pyridine in a 4-mL glass screw-top vial, followed by 700 μL of hexamethyldisilazane, 50 μL of 0.5% heptanoic acid in pyridine as an internal standard, and 80 μL of trifluoracetic acid. The mixture was shaken for 30 s to form a single layer and heated at 70 °C for 2 h.

GC. Liquid samples were analyzed using a J&W (Folsom, CA) 30-m × 0.25-mm-i.d. DB-5 silica capillary column with a 1-m × 0.25-mm-i.d. fused silica guard column in a Hewlett-Packard (Palo Alto, CA) 6890A gas chromatograph with Hewlett-Packard 3365 Series II ChemStation data analysis software. For butylated samples, the column temperature remained at 50 °C for 10 min, followed by a 2.5 °C min^{-1} increase to 150 °C, remaining at that temperature for 10 min. After each run, the column was rapidly taken to 300 °C to expel any remaining high-boiling material. The flow rate of the helium carrier gas and split ratio were 1.2 mL min^{-1} and 74.3, respectively, while the injector and flame ionization detector temperatures were 265 °C. The same program was followed for TMS-derivatized samples, except that after 10 min at 150 °C, the temperature was increased at 20 °C min^{-1} to 280 °C and was held there for 10 min. Samples of 3 μL derivatized by each method were injected with a 10-μL glass syringe, flushed with acetone, and completely washed with the new analyte before each injection.

Peak areas of liquid samples were divided by the heptanoic acid peak area in each chromatogram and then multiplied by the mean heptanoic acid peak area in all chromatograms. Adjusted peak areas from two chromatograms derived from the two butylated samples with the same starting compositions and the same incubation times were averaged. A systematic error, rather than random errors, between the first and second samples with all three starting materials and the control occurred, leading to the use of averaging. Similarly, one chromatogram from each of two TMS-derivatized samples was obtained, and their adjusted peak areas were averaged.

Chromatographic data from TMS-derivatized samples were fitted by exponential decay equations. Those from butylated samples were fitted by hyperbolic equations with initial concentrations C_{i0} and constants $C_{i\max}$ and A_i, where t is incubation time and i varies with both product and added material:

$$C_i = C_{i0} + \frac{C_{i\max} t}{A_i + t} \quad (1)$$

Conclusions were inferred from curves obtained from the above nonlinear regression techniques rather than from individual data points. Therefore, tests of significance were based on the dependent variables and their standard errors for each curve. Differences between two values of C_{i0} were deemed significant when the sum of one C_{i0} and its standard error did not overlap the value of the other C_{i0}. Differences in hyperbolic curves were judged by comparing two values of C_i when $t = \infty$, giving $C_i = C_{i0} + C_{i\max}$. They were deemed significant when the sum of one C_i and the standard errors of its C_{i0} and $C_{i\max}$ values did not overlap the value of the other C_i, and very significant when one sum did not overlap the other sum.

Fermentation with Propylene Glycol for Gas Sampling. Separate samples containing rumen fluid with anaerobic dilution buffer, pH 6.8, and cysteine were treated with 25 mM propylene glycol and were incubated for 4 h at 37 °C in 20-mL twister headspace vials (Gerstel) containing twister stir bars to capture gas for GC−MS−O analysis.

GC−MS−O Analysis. Qualitative analysis of samples absorbed to twister stir bars was performed using a Gerstel (Baltimore, MD) TDS-2 thermodesorption unit with an autosampler mounted on an Agilent (Wilmington, DE) 6890/5973N gas chromatograph−mass spectrometer (GC−MS) equipped with a Gerstel GC−O sniffing port, a Gerstel CIS 4 programmed temperature vaporizer (PTV) inlet, and an Agilent HP-FFAP column (30 m × 0.25 mm × 0.25 μm) using helium eluent at 1.3 mL min^{-1} constant flow.

Thermodesorption occurred in splitless mode, with the temperature held at 25 °C for 0.5 min and then increased linearly to 250 °C at 3 min, and with a transfer line temperature of 275 °C. A glass bead/Carbotrap C packed inlet was used in the PTV with solvent vent mode, with temperature held at −80 °C for 0.2 min and then increased linearly to 280 °C at 3 min, and with a vent flow of 40 mL min^{-1} and a purge split flow of 10 mL min^{-1}. The oven temperature was 20 °C until 0.5 min, followed by a linear increase to 240 °C at 10 °C min^{-1}, with a final hold of 5 min.

Sample effluent was split 8:1 between exhaust and the analytical column, while the analytical GC effluent was split 2:1 between the sniffing port and mass spectrometer, respectively. The transfer line of the GC−O sniffing port was held at 250 °C, and humidified air was added in the sniffing port at 100 mL min^{-1}. Eluting odor-active compounds were recorded on a Gerstel ODP2 olfactory intensity device. The MS transfer line and source temperatures were 240 and 250 °C, respectively. Mass spectra ($40 < m/z < 350$) were collected at 4.58 scans/s. Compounds were identified using mass spectra and retention times of reference standards and by matches of mass spectra of >90% with the NIST98 library (NIST, Gaithersburg, MD) and with the results of Boelens et al. (*23*).

Olfactometry Analysis (Frequency Response). GC−O frequency analysis was performed using GC−sniff technology (*24*). Eight nontrained volunteer panelists analyzed chromatographed rumen fluid treated with propylene glycol, while four volunteer nontrained panelists analyzed rumen fluid control samples, all samples being taken after 4 h of incubation. The average age of the panelists was 32 years (range 21−51). Each panelist analyzed only one sample per session, and verbal descriptors were recorded either manually or on wave files connected to each odor event using Gerstel odor port software. Only compounds described as having an onion/garlicky aroma and associated exclusively with the propylene glycol-treated rumen fluid were considered as major odorants.

Onion-like Odor-Bearing Compounds. Propanal solution was mixed with H_2S to produce onion-like odors. It was prepared by diluting 250 μL of propanal (98% pure by GC, Fluka, Steinheim, Switzerland) with 100 mL of HPLC-grade water (Burdick & Jackson, Morristown, NJ). Solutions of methylthiirane (96% pure by GC) and dipropyl disulfide (98% pure by GC, both Aldrich, Milwaukee, WI) were prepared by adding 100 μL of either compound to 50 mL of HPLC-grade water. The H_2S was created by placing 2.2 g of ZnS (Fisher, Pittsburgh, PA) into a 500-mL Erlenmeyer sidearm flask connected on one end to a 10-L Tedlar bag and on the other end to the house vacuum system. The flask was then evacuated, and 50 mL of Fisher ACS-certified concentrated HCl was added dropwise. The approximately 0.5 L of H_2S gas that formed was collected in the Tedlar bag.

Table 1. Parameters (Eq 1) for Formation of Fatty Acids

fatty acid	added material	C_{i0} (mM)	$C_{i\max}$ (mM)	A_i (h)
acetate	*R*-propylene glycol	36.1 ± 3.8	33.4 ± 9.8	8.20 ± 6.61
	S-propylene glycol	39.0 ± 5.1	16.5 ± 7.7	2.03 ± 3.25
	glycerol	38.4 ± 5.0	14.6 ± 7.7	2.30 ± 4.06
	none	36.1 ± 1.6	70.1 ± 13.9	23.0 ± 9.1
propionate	*R*-propylene glycol	17.6 ± 1.7	88.8 ± 17.3	28.6 ± 10.2
	S-propylene glycol	20.2 ± 2.5	133 ± 95	54.9 ± 56.8
	glycerol	17.5 ± 2.2	21.6 ± 4.4	5.31 ± 3.14
	none	17.6 ± 1.8	25.5 ± 5.2	9.44 ± 5.12
butyrate	*R*-propylene glycol	10.5 ± 1.3	13.8 ± 3.1	7.65 ± 4.82
	S-propylene glycol	11.8 ± 1.3	9.24 ± 2.44	4.80 ± 3.77
	glycerol	10.7 ± 1.2	21.5 ± 3.6	9.73 ± 4.27
	none	11.6 ± 1.2	22.0 ± 5.7	14.6 ± 8.9
valerate	*R*-propylene glycol	2.63 ± 0.21	10.1 ± 1.3	18.2 ± 5.0
	S-propylene glycol	2.71 ± 0.48	11.4 ± 2.7	17.2 ± 9.0
	glycerol	2.23 ± 0.38	11.2 ± 1.3	11.0 ± 3.2
	none	2.78 ± 0.45	6.69 ± 1.33	9.70 ± 5.12
caproate	*R*-propylene glycol	1.32 ± 0.05	2.20 ± 0.10	5.46 ± 0.74
	S-propylene glycol	1.38 ± 0.14	2.25 ± 0.29	4.84 ± 1.94
	nlycerol	1.33 ± 0.16	3.78 ± 0.39	7.59 ± 2.17
	none	1.50 ± 0.16	2.61 ± 0.41	8.21 ± 3.52

Propanal solution (5 mL) was added to a 20-mL Gerstel twister headspace vial along with a twister stir bar in a glass insert. The headspace vial was sealed, and 20 mL of gas was removed by syringe. Samples of 1, 5, or 10 mL of H_2S were introduced into the headspace vial, vortexed for 1 min, and incubated for 1 h at 37 °C. After incubation, the twister stir bars were removed and analyzed by GC−MS. In addition, 5 mL of either methylthiirane or dipropyl disulfide solution was added to the 20-mL twister headspace vial along with a twister stir bar in a glass insert and 20 mL of H_2S. The vials were incubated for 2 h at 37 °C and analyzed by GC−MS. Control solutions of methylthiirane and dipropyl disulfide and equal-volume solutions of the two were incubated for 1 day at 37 °C with twister stir bars and analyzed by GC−MS. All GC−MS procedures followed GC−MS−O procedures, except that no panelists monitored the olfactometry port.

RESULTS

Propylene Glycol and Glycerol Reacted with Rumen Fluid. *R*- and *S*-propylene glycol and glycerol, all originally 25 mM, were metabolized by rumen fluid. The first two decreased more or less exponentially and reached zero between 8 and 24 h. There was no significant difference between them. Glycerol disappearance was also roughly exponential, one-fifth of the original concentration remaining after 24 h. Approximately 0.25 mM glycerol was found initially in samples incubated with no added substrate or with *R*- and *S*-propylene glycol; its concentration remained about the same throughout the fermentations. Lactic acid appeared after 2 h when 25 mM glycerol was initially added. It increased rapidly through 8 h and was approximately 1.5 mM at 24 h.

Acetate, propionate, butyrate, valerate, and caproate concentrations, in generally decreasing order, increased throughout all fermentations (**Figure 1**), in all cases being at least moderately well fitted by eq 1. Furthermore, average initial concentrations of each of the five products did not vary significantly over the four different pairs of samples, as expected since all of these samples came from the same initial sample (**Table 1**).

Acetate concentration reached its highest level in the control samples, suggesting that added propylene glycol and glycerol somewhat suppress acetate formation. Propionate concentrations were highest when *R*- and *S*-propylene glycol were added, indicating that they were converted to propionate during the fermentation. Conversely, adding either form of propylene glycol led to lower butyrate concentrations. Glycerol addition yielded higher valerate and caproate concentrations. In general, there

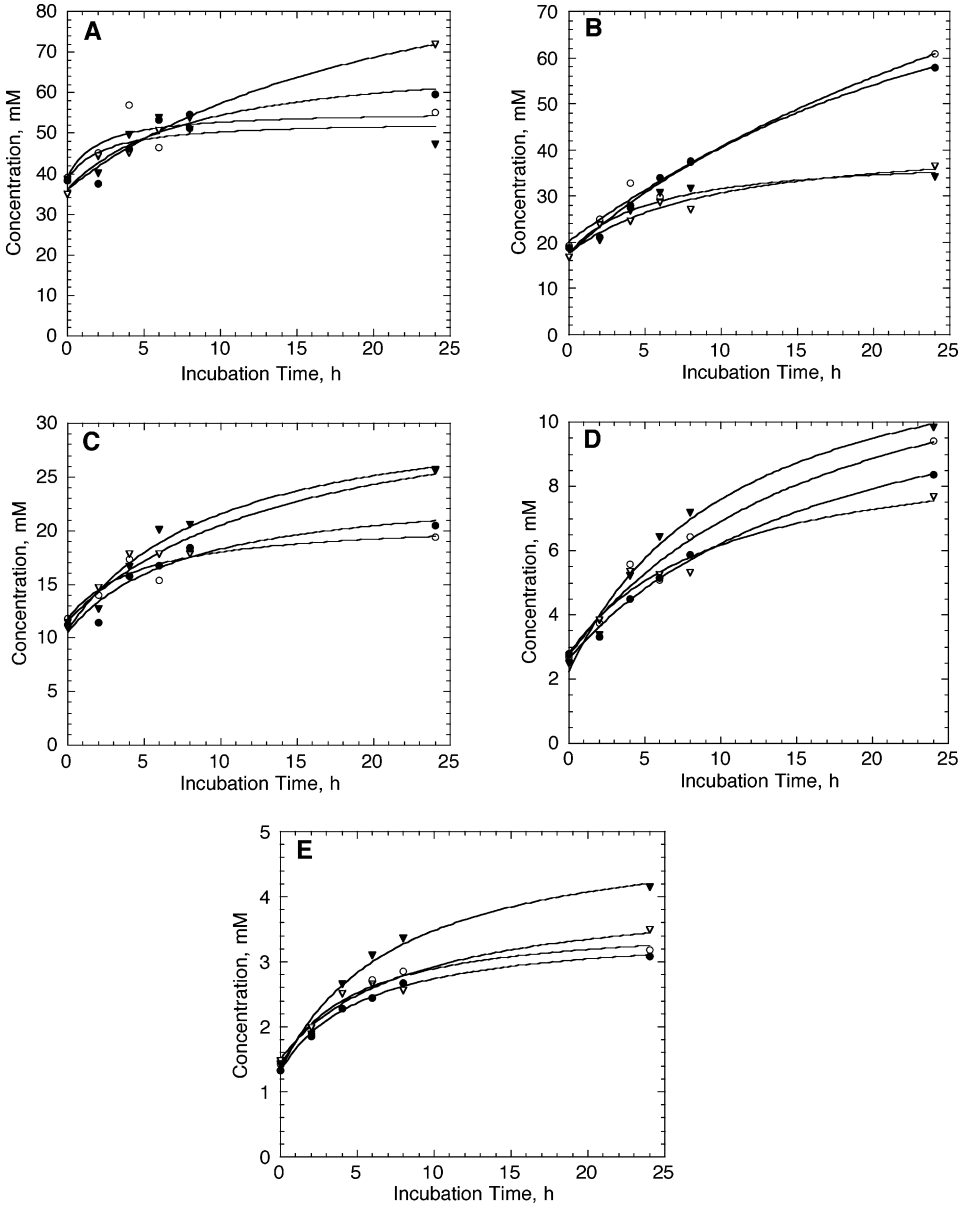

Figure 1. Changes of concentration with incubation time of (**A**) acetate, (**B**) propionate, (**C**) butyrate, (**D**) valerate, and (**E**) caproate. Added substrates: *R*-propylene glycol (●), *S*-propylene glycol (○), glycerol (▼), and none (▽).

was no significant difference between *R*- and *S*-propylene glycols in terms of their influence on product formation (**Table 1**).

GC−O of Products Formed by Propylene Glycol Reacted with Rumen Fluid. Rumen fluid treated with propylene glycol and incubated for 4 h had an overall odor described as strongly onion/garlicky by five of the eight panelists and as slightly onion-like with strong "rumen" odor by the other three. This material had an average response of 33 odor-active compounds, of which 12 on average were described by panelists as either onion-like or garlicky. In the control rumen fluid samples, panelists detected an average of 19 odor-active compounds, of which only six on average were described as either onion-like or garlicky. **Figure 2** shows GC−MS chromatograms of a twister stir bar alone, control rumen fluid, and rumen fluid treated with propylene glycol, while **Figure 3** gives a GC−MS chromatogram and a GC−O aromagram for rumen fluid treated with propylene glycol.

The two criteria used to evaluate the importance of a compound's contribution to the overall onion/garlicky odors were that aromas had to be described as onion/garlicky at least once and that compounds could not be detected in any of the rumen control samples. **Table 2** shows the results of the GC−sniff analysis of rumen fluid treated with propylene glycol. On the basis of nasal impact frequency (NIF) analysis, 1-propanethiol **(1)** and methylthiirane **(3)** are the major contributors to the onion/garlicky aroma, since these compounds were detected by all panelists. In addition, 1-(methylthio)propane **(2)**, 2,4-dimethythiophene **(4)**, and dipropyl disulfide **(6)** appear to contribute significantly to the onion-like odor, since they were detected by six out of eight panelists via GC−O and were detected in all GC−MS chromatograms. Other compounds of note are 1-(methylthio)-1-propanethiol **(5)**, 1-(propylthio)-1-propanethiol **(7)**, dipropyl trisulfide **(8)**, and isomers of 3,5-diethyl-1,2,4-trithiolane **(9)**, which were detected by four to six panelists and are present in most but not all GC−MS chromatograms. Both 2-ethyl-1,3-dithiane (**10**) and 2,4,6-triethyl-1,3,5-trithiane **(11)** were detected by fewer than three panelists and are considered very minor contributors to the overall onion/garlicky odor. Identifications of 1-propanethiol, 1-(methylthio)-

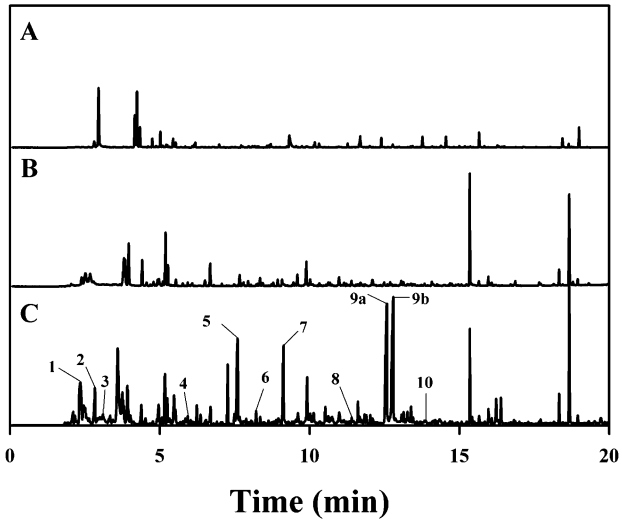

Figure 2. GC–MS chromatograms of (**A**) twister stir bar, (**B**) twister stir bar headspace of rumen fluid, and (**C**) twister stir bar headspace of rumen fluid treated with propylene glycol. Identified compounds are (**1**) 1-propanethiol; (**2**) 1-(methylthio)propane; (**3**) methylthiirane; (**4**) 2,4-dimethylthiophene; (**5**) 1-(methylthio)-1-propanethiol; (**6**) dipropyl disulfide; (**7**) 1-(propylthio)-1-propanethiol; (**8**) dipropyl trisulfide; (**9a**,**b**) isomers of 3,5-diethyl-1,2,4-trithiolane; and (**10**) 2-ethyl-1,3-dithiane.

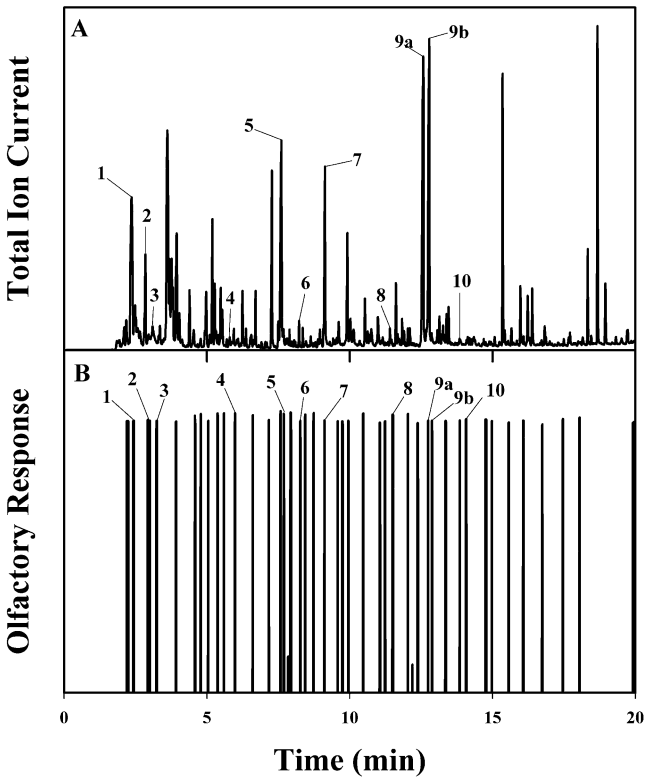

Figure 3. (**A**) GC–MS chromatograms and (**B**) GC–O aromagrams for rumen fluid treated with propylene glycol. Identified compounds having onion/garlicky odors are (**1**) 1-propanethiol; (**2**) 1-(methylthio)propane; (**3**) methylthiirane; (**4**) 2,4-dimethylthiophene; (**5**) 1-(methylthiol)-1-propanethiol; (**6**) dipropyl disulfide; (**7**) 1-(propylthio)-1-propanethiol; (**8**) dipropyl trisulfide; (**9a**,**b**) isomers of 3,5-diethyl-1,2,4-trithiolane; (**10**) 2-ethyl-1,3-dithiane.

propane, methylthiirane, 2,4-dimethylthiophene, and dipropyl disulfide were based on reference standards and MS fragmentation patterns, while those of dipropyl trisulfide, 3,5-diethyl-1,2,4-trithiolane, and 2-ethyl-1,3-dithiane were based on NIST library searches of greater than 90% match (**Table 2**). Identification of 1-(methylthio)-1-propanethiol, 1-(propylthio)-1-propanethiol, and 2,4,6-triethyl-1,3,5-trithiane was based on fragmentation patterns of these compounds (**Figure 4**) compared to those reported by Boelens et al. (*23*). In addition to the compounds listed in **Table 2**, dimethyl disulfide and dimethyl trisulfide were also described in the literature as having onion/garlicky aromas and were identified in most of the GC−MS chromatograms of propylene glycol-treated samples, but they were also detected in rumen control samples and therefore will not be further considered here.

Propanal Reacted with H_2S. Table 2 lists the organosulfur compounds formed both in rumen fluid treated with propylene glycol and by reaction of propanal with H_2S (using molar ratios from 1:4 to 2.5:1) in an aqueous environment. The latter reaction produced mainly methylthiirane after 1 h of incubation, along with 1-propanethiol (data not shown). Other compounds detected included dipropyl disulfide, dipropyl trisulfide, 2,4-dimethylthiophene, and 2-ethyl-1,3-dithiane. The heterocyclic compounds 2,4,6-triethyl-1,3,5-trithiane and 3,5-diethyl-1,2,4-trithiolane were also detected, with levels of the former rising with increasing incubation times and aqueous system acidities. Potentially two other dithiane structures formed, as evidenced by their parent mass (148 *m*/*z*) and main fragmentation ion (74 *m*/*z*). However, neither of these compounds, when subjected to NIST library searches, had matches of >50%, nor did Boelens et al. (*23*) report similar types of compounds.

Methylthiirane and Dipropyl Disulfide Reacted with H_2S. Two of the main products of the propanal−H_2S reaction, methylthiirane and dipropyl disulfide, were reacted further with H_2S. Methylthiirane yielded all the same products formed by the propanal−H_2S reaction. In addition, methylthiirane and H_2S also formed 1,2-propanedithiol, which was not detected either in the propanal−H_2S reaction or in rumen fluid treated with propylene glycol. Additional treatment of dipropyl disulfide with H_2S did not result in additional compounds being formed, except for dipropyl trisulfide, a known oxidation product of dipropyl disulfide, which was a contaminant in the dipropyl disulfide standard. Methylthiirane and dipropyl disulfide were both stable in an aqueous environment when no H_2S was present. Based on results from propylene glycol-treated rumen fluid and the propanal−H_2S reaction, the overall hypothetical pathways forming the various organosulfur compounds from propylene glycol-treated rumen fluid appear in **Figure 5**.

DISCUSSION

There is a significant difference in the fermentation of the control samples gathered here and that of those from our previous work (*20*). The earlier control samples, from rumen fluid with no added substrates, showed no changes in acid concentrations with time. In the present control samples, from rumen fluid with no propylene glycol or glycerol added, all acid concentrations increased with time. This difference is attributed to the much richer ration fed to the donor cattle in this work, and to the fact that they were fed before rumen samples were obtained. This required that increases or decreases of acid concentrations in samples with added propylene glycol or glycerol be measured in reference to the acid concentrations in the control samples at the same incubation times.

Propylene glycol added to rumen fluid in moderate amounts was more quickly metabolized than glycerol added at the same concentrations, but lactate accumulation may have slowed microbial activity in glycerol fermentations. Furthermore, the rumen microbiota treat *R*- and *S*-propylene glycol equally, both in metabolizing it and in producing acids.

Table 2. Detected Sulfur Compounds and Their Odor Characteristics

	compound	t_R[a] (min)	detection R[b]	P[c]	M[d]	ID	intensity $\times 10^6$ [e]	NIF[f] (%)	odor description GC–O panel	literature
1	1-propanethiol	2.3	+	+	+	RS[g]	5.86	100	onion, garlicky	cabbage, penetrating and diffusive, sulfurous very powerful[h]
2	1-(methylthio)propane	2.8	+	–	–	RS	2.16	75	onions	–
3	methylthiirane	3.1	+	+	+	RS	3.26	100	onions	cabbage, pungent[i]
4	2,4-dimethylthiophene	5.8	+	+	+	RS	0.25	75	cut grass, onion, garlicky	garlicky[i]
5	1-(methylthio)-1-propanethiol	7.6	+	–	–	MS[j]	3.80	63	strong onions, garlic	allium, meat, onion[h]
6	dipropyl disulfide	8.3	+	+	+	RS	0.37	75	pungent, tar, onion	diffusive, nonlachrymatory, of garlic/onion type, very penetrating and powerful[h]
7	1-(propylthio)-1-propanethiol	9.1	+	–	–	MS	0.76	50	sweet onions	allium, black currant, onion[h]
8	dipropyl trisulfide	11.5	+	+	+	MS	0.06	50	sour, onion, sweating	garlic, onion, penetrating and repulsive, very powerful and diffusive[h]
9a	3,5-diethyl-1,2,4-trithiolane[k]	12.5	+	+	+	NIST[l]	6.50	75	onions	garlic[h]
9b	3,5 diethyl-1,2,4-trithiolane[k]	12.8	+	+	+	NIST	7.19	75	onions	garlic[h]
10	2-ethyl-1,3-dithiane	13.9	+	+	+	NIST	0.31	37	onions	–
11	2,4,6-triethyl-1,3,5-trithiane	16.5	+	+	+	MS	n.a.[m]	13	onions	allium, garden cress, green, onion[h]

[a] Retention time. [b] Rumen fluid treated with propylene glycol. [c] Propanal solution reacted with H_2S. [d] Methylthiirane solution reacted with H_2S. [e] Intensity average area count associated with compounds detected in rumen fluid treated with propylene glycol. [f] Nasal impact frequency response. [g] Reference standard. [h] Reference 25. [i] Reference 26. [j] Mass spectrum matching compounds reported by Boelens et al. (*23*). [k] Two isomers of 3,5-diethyl-1,2,4-trithiolane, one *cis* and the other *trans*. [l] Mass spectrum of compound had an NIST library match of greater than 90%. [m] Not applicable due to only one detection in rumen fluid.

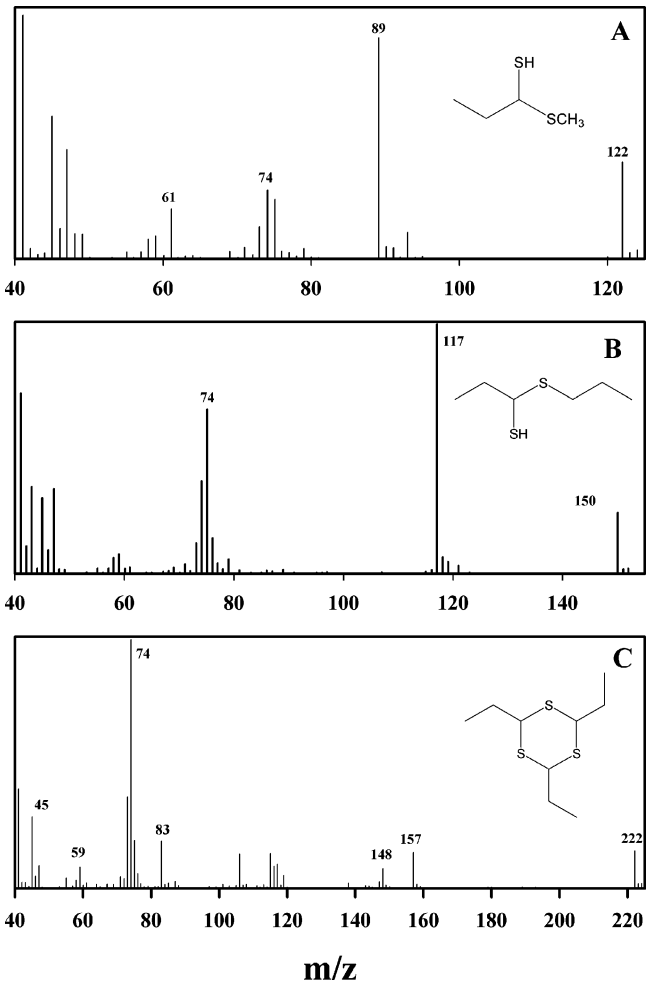

Figure 4. Mass spectra of (**A**) 1-(methylthio)-1-propanethiol, (**B**) 1-(propylthio)-1-propanethiol, and (**C**) 2,4,6-triethyl-1,3,5-trithiane.

As found previously in both *in vivo* and *in vitro* studies (*15*, *17*–*19*), we observed that the propionate-to-acetate ratio increased when propylene glycol and glycerol were added to rumen fluid. Addition of propylene glycol sharply decreased formation of butyrate; we did not observe increased butyrate formation with added glycerol, unlike in earlier work (*17*). Adding either propylene glycol or glycerol increased valerate, but only glycerol addition increased caproate.

Identifying by GC–MS–O analysis the sulfur-containing materials emitted by rumen fluid incubated with propylene glycol and having onion/garlicky odors is of high interest, given the potential for sulfur toxicity when feeding cattle with distillers grains from ethanol production (*27*), and given that high doses of propylene glycol can cause symptoms of toxicity in cattle that are similar to those of classical sulfur toxicity. All 12 compounds identified share the propyl backbone from propylene glycol in some form (**Figure 5**). Although most of the propylene glycol is fermented to propionic acid under anaerobic conditions (*28*), it appears that the odors are the result of a fermentation pathway having propanal formed by dehydratase catalysis (*29*, *30*), a minor pathway from propylene glycol (**Figure 5**). Propanal formation from propylene glycol fermentation has been previously observed in rumen samples (*31*). Reaction of propanal with either free H_2S or methanethiol is thought to produce the various organoleptic compounds detected (*23*, *32*). In fact, bulk air samples from reaction mixtures of propanal and H_2S have been described in our laboratory and by others (*23*) as having a strong fresh onion-like odor by direct olfactory perception.

The initial step in forming the various organosulfur compounds is believed to be formation of the analogous *gem*-diol or hemiacetal structures from the reaction of the propanal C1 atom with either H_2S or methanethiol, respectively. In this study, methylthiirane (**3**) along with some 1-propanethiol (**1**) were the main products of propanal, which reacted with H_2S in pH 7 water. In fact, ring closure to give thiiranes is relatively easy due to the lower activation energy of formation and the close proximity of the nucleophilic atom to the electrophilic site (*33*). Shagun et al. (*34*) reported that aliphatic *gem*-dithiols form mainly thiirane derivatives, while Zheng et al. (*35*) found high

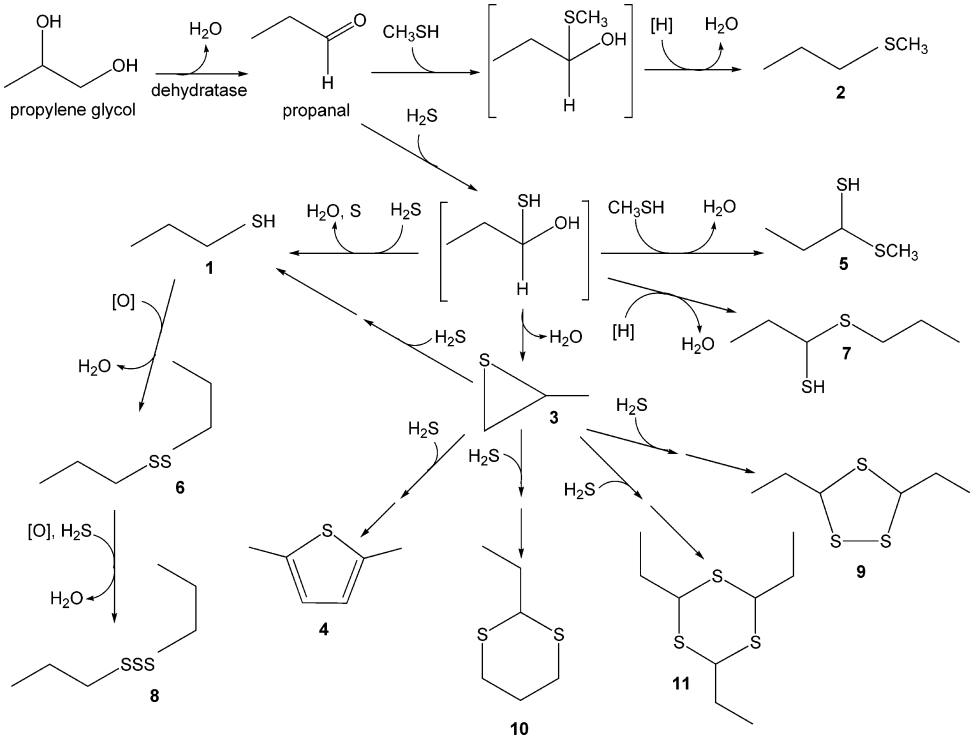

Figure 5. Proposed pathways to produce compounds having onion/garlicky odors from propylene glycol added to rumen fluid. Identified compounds are (**1**) 1-propanethiol; (**2**) 1-(methylthio)propane; (**3**) methylthiirane; (**4**) 2,4-dimethylthiophene; (**5**) 1-(methylthio)-1-propanethiol; (**6**) dipropyl disulfide; (**7**) 1-(propylthio)-1-propanethiol; (**8**) dipropyl trisulfide; (**9**) 3,5-diethyl-1,2,4-trithiolane; (**10**) 2-ethyl-1,3-dithiane; and (**11**) 2,4,6-triethyl-1,3,5-trithiane.

levels of thiirane formation in Furaneol reacted with cysteine, a precursor to H_2S in biological systems. The formation of the corresponding thiols 1-propanethiol (**1**) and 1-(methylthio)-propane (**2**) should also occur due to the instability of the analogous *gem*-diol and hemiacetal structures and the strength of the sulfur nucleophiles compared to hydroxyl groups. Mechanisms to form such intermediates and to produce thiols from these intermediates have been previously proposed (*36*, *37*). Structure **5** could be derived from the reaction of methanethiol with the analogous *gem*-diol, while **7** could result from condensation of the *gem*-diol. Boelens et al. (*23*) detected both **5** and **7** after reacting propanal with both methanethiol and H_2S.

Reaction of methylthiirane (**3**) with H_2S leads to the formation of all the other organosulfur compounds identified here. Thiiranes are reactive in the presence of strong nucleophiles such as H_2S, leading to their ring opening **(***38*, *39*) and the formation of **1** (**Figure 5)**, which can be oxidized to form **6** and **8**. Structures **9**, **10**, and **11** are analogous to ring compounds found when thiirane is reacted with H_2S (*40*).

Isomers of **9** were among the main organosulfur compounds formed in rumen fluid treated with propylene glycol (**Table 2**), but when propanal or methylthiirane was reacted with H_2S, these isomers were detected at low levels. Oxidation of **7** could result in forming **9**, but given the amount of **7** formed compared to **9** (**Table 2**), its contribution to formation of **9** would be minor. Previously, Boelens et al. (*23*) reported that **9** formed upon exposing bis(1-thiopropyl) sulfide to an oxygenated environment. In our study, we never detected bis(1-thiopropyl) sulfide in any medium, but this may be a result of our twister stir bars being stored under aerobic conditions prior to analysis. Consequently, **9** may be formed through several different pathways. Compound **11** was detected at low levels in all media tested, but under acidic conditions this compound formed readily, as previously noted by Boelens et al. (*23*).

The identity of the various onion/garlicky odors also clarifies the toxicological effects observed from ingesting large doses of propylene glycol. In addition to gastrointestinal tract absorption, it is known that volatile sulfur compounds are removed from the rumen via gas exchange with the lungs, where toxic sulfur gases are readily absorbed through the pulmonary mucosa. The propyl sulfides identified here have also been detected in onions, leeks, and garlic (*41*−*44*), and it is believed that propyl sulfides in onions are partly responsible for their toxic effects on ruminants (*45*−*49*). Symptoms of ruminants that have ingested large quantities of onions include weakness, poor appetite, depression, and ataxia, many of which are also observed for ruminants and other herbivores given large doses of propylene glycol (*5*, *13*−*15*). This would indicate some type of hemolytic anemia and potentially Heinz body anemia, a condition in which hemoglobin is denatured via oxidative stress (*49*, *50*). In fact, hemolytic anemia has been induced in rats given propenyl disulfides (*50*). The toxicological literature is in general agreement on the toxicity of these sulfur-containing compounds.

It is hoped that this work sheds further light on the metabolism of propylene glycol and glycerol in the rumen, especially on the origin and identity of the sulfur-containing gases exhaled by ruminants fed propylene glycol.

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