Determination of Henry's constant, the dissociation constant, and the buffer capacity of the bicarbonate system in ruminal fluid

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ABSTRACT

Despite the clinical importance of ruminal acidosis, ruminal buffering continues to be poorly understood. In particular, the constants for the dissociation of H_2CO_3 and the solubility of CO_2 (Henry's constant) have never been stringently determined for ruminal fluid. The pH was measured in parallel directly in the rumen and the reticulum in vivo, and in samples obtained via aspiration from 10 fistulated cows on hay- or concentrate-based diets. The equilibrium constants of the bicarbonate system were measured at 38°C both using the Astrup technique and a newly developed method with titration at 2 levels of partial pressure of CO_2 (pCO_2 ; 4.75 and 94.98 kPa), yielding mean values of $0.234 \pm 0.005 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$ and 6.11 ± 0.02 for Henry's constant and the dissociation constant, respectively ($n/n = 31/10$). Both reticular pH and the pH of samples measured after removal were more alkaline than those measured in vivo in the rumen (by $\Delta\text{pH} = 0.87 \pm 0.04$ and 0.26 ± 0.04). The amount of acid or base required to shift the pH of ruminal samples to 6.4 or 5.8 (base excess) differed between the 2 feeding groups. Experimental results are compared with the mathematical predictions of an open 2-buffer Henderson–Hasselbalch equilibrium model. Because pCO_2 has pronounced effects on ruminal pH and can decrease rapidly in samples removed from the rumen, introduction of a generally accepted protocol for determining the acid-base status of ruminal fluid with standard levels of pCO_2 and measurement of base excess in addition to pH should be considered.

Key words: rumen, bicarbonate, dissociation constant, Henry's constant

INTRODUCTION

Acute and subacute ruminal acidosis is a fermentational disorder afflicting ruminants with symptoms can negatively affect milk fat content, feed intake, and milk yield. The condition affects some 10 to 40% of dairy cows at least once in a lifetime and is associated with a low ruminal pH, inflammation of the ruminal epithelium, and loss of ruminal barrier function (Enemark, 2008; Plaizier et al., 2012). Efflux of pathogens and their toxins into the portal circulation follows (Plaizier et al., 2008). Symptoms include systemic immunological complications with lameness, liver abscesses, and dehydration due to ruminal bloating that can be lethal (Kleen et al., 2003; Plaizier et al., 2008).

Early diagnosis is crucial because animals can recover after shifting dietary balance away from starches and toward physically effective neutral detergent fiber to stimulate chewing activity and the production of salivary buffers such as NaHCO_3 (Allen, 1997; Mertens, 1997; Yang and Beauchemin, 2006; Zebeli et al., 2012; Maulfair et al., 2013). Although correlations exist between SARA and parameters such as a drop in milk fat or a rise renal net acid excretion, these criteria have yet to replace ruminal pH as the leading diagnostic tool (Enemark, 2008). Investigators have suggested various pH thresholds ranging from pH <5 to pH <5.2 for the acute form of the disease, whereas a range of pH <5.5 to pH <6.0 or even higher has been suggested for SARA (Duffield et al., 2004; Krause and Oetzel, 2006; Plaizier et al., 2008). Depending on the study, these thresholds can refer to measurements at a certain time point, the daily means, or the nadir of a continuous measurement. Even if the procedure is identical, the pH ranges given are clearly too large to satisfy the clinician in the field who has to make a decision on whether dietary intervention is necessary or not (Li et al., 2012). Accordingly, various other parameters for identifying animals or herds at risk are being discussed, which include the concentrations of ruminal acetic, propionic,

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isobutyric, butyric, and caproic acids, lactate, and ammonia (Krause and Oetzel, 2005; Bramley et al., 2008).

A parameter that is curiously missing from this rising list is the partial pressure of CO₂ (pCO₂) in the rumen, which is known to vary between 36.7 and 66.5 kPa (Turner and Hodgetts, 1955; McArthur and Miltimore, 1961; Kölling, 1974; Counotte et al., 1979) and can be expected to drop sharply after removal of a sample, in particular when pressure drops during aspiration. Spurious changes of this nature can thus be expected to add to the poor outcomes of attempts at identifying precise thresholds for the onset of ruminal acidosis.

When trying to assess the magnitude of a variation in pCO₂ on ruminal pH, another omission emerges: the only available value for the dissociation constant (pK_{app}) of H₂CO₃ in ruminal fluid was determined decades ago at an inappropriate temperature (25°C; Turner and Hodgetts, 1955) and using a value for the solubility of CO₂ (α) that was obtained in 1917 in aqueous solution (Van Slyke and Cullen, 1917). In one of the very few more recent detailed studies of the issue (Counotte et al., 1979), the authors concluded that “bicarbonate will not play an important role as a base, until the pH in the rumen is 6.25 or lower.” Made in the context of an otherwise exemplary study, this statement suggests that the authors are erroneously treating the rumen as a closed buffer system in which buffer capacity is maximal around the pK value of the bicarbonate system.

Several studies since have clarified that due to absorption of CO₂ from the rumen and regular eructation, the bicarbonate system of the rumen has to be regarded as an open buffer system comparable to that of the blood (Allen, 1997; Kohn and Dunlap, 1998). Protons react with HCO₃[−] to form H₂O and CO₂ ($H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$). Because CO₂ does not accumulate in the system but escapes, the bicarbonate system buffers strongly at neutral pH and beyond. A further problem that emerges when trying to understand ruminal buffering is that equations solving the underlying mixed SCFA-HCO₃[−] buffer problem are lacking in the literature. These have been now been derived and are given in the Appendix of this study.

A major question that remains is whether the values for Henry's constant (α) and the dissociation constant (pK) that were determined for blood plasma (Andersen, 1962; Segel, 1976) can be applied to ruminal fluid. Both α and the pK of the bicarbonate system are known to vary not only with the osmolarity and the temperature, but also with protein content and various other parameters (Heisler, 1986). Given that the effect of short-chain fatty acids (SCFA) on both constants has never been studied, a rigorous determination of both constants for fluid from the rumen appears overdue. In

the current study of bovine ruminal solution, Henry's constant α and the pK of H₂CO₃ are determined using a classical variation of the Astrup technique. These results are confirmed by those obtained via a novel titration method developed in the course of this study. Finally, the concept of base excess was adapted to allow a better evaluation of the acid-base status of ruminal fluid.

MATERIALS AND METHODS

Care and Use of Animals

All experiments were performed in accordance with German laws for the protection of animals [permits L0016/09 (Berlin) and 33.12-42502-04-14/1501 (Braunschweig)].

Animals and Sampling

A first set of samples was obtained from 2 fistulated cows (**h1** and **h2**) that were kept in tethered housing on straw at the faculty of veterinary medicine in Berlin. Initially, the animals received a hay-only diet at 0730 and 1500 h and water ad libitum. These animals will be referred to as hay/silage-fed in what follows because the animals received small amounts of grass silage in addition to hay toward the end of the experimental period.

The 2 hay/silage-fed cows were sampled twice weekly for a period of 3 wk. On experimental days, samples were taken immediately before the morning meal and approximately 4 h afterward (0700 and 1100 h) so that, in total, 12 samples were obtained from each of the 2 cows.

Before sampling, a 3-way stopcock was fitted to the fistula plug, allowing the measurement of intraruminal pressure using a differential pressure measurement device (Testo 510, Testo, Letzkirch, Germany). Negative basal values of -0.71 ± 0.06 kPa ($n = 24$) inside the rumen were observed versus barometric pressure outside, which alternated with short pressure bouts above the barometric level reflecting eructation.

After opening the fistula, intraruminal pH was measured approximately 10 to 15 cm below liquid level using a portable pH meter (pH-Meter 1140, Mettler Toledo, Gießen, Germany) calibrated at 37°C with standard buffer (pH 4 and 7) immediately before use. Subsequently, approximately 200 mL of ruminal fluid were obtained after insertion of an oro-ruminal sampling device into the rumen through the ruminal fistula. The pH of this sample was also recorded. Fluid aliquots were immediately frozen in liquid nitrogen to stop fermentation processes and stored at −20°C.

For comparison, one sample each was obtained from 8 fistulated cows in late lactation (Federal Research Institute for Animal Health, Braunschweig, Germany), referred to as concentrate/silage-fed in what follows. The animals were kept in housing with free stalls on a performance-oriented diet (60% maize and 40% grass silage based on DM content) and received water *ad libitum*. Each cow additionally received 4 kg of concentrate feeding per day via an automated feeding system with radio frequency technology for identification of individual animals. In addition to sampling of ruminal fluid through the fistula, these animals were equipped with an electronic pH sensor placed in the rumenoreticulum to measure pH and temperature (smaXtec Animal Care GmbH, Graz, Austria), calibrated as suggested by the manufacturer. Data obtained during the hour before sampling were averaged. Otherwise, sampling and measurement of ruminal pH was performed as described above.

Measurement of SCFA Concentrations

Analysis of SCFA was performed by gas chromatography as described previously, yielding values for acetic, propionic, i-butyric, n-butyric, i-valeric, n-valeric, and total SCFA (Pieper et al., 2012).

Determination of Henry's Constant α , the pK_{app} , and the Buffer Capacity β_{NB} of Ruminal Fluid

The solubility of CO_2 (α) in ruminal fluid and the apparent dissociation constant pK_{app} of ruminal fluid were determined using 2 separate approaches: a classical variation of the Astrup technique (Heisler, 1986) and a novel titration technique.

In brief, each sample of ruminal fluid was thawed and centrifuged ($3,200 \times g$, 5 min, ambient temperature) to minimize feed residues and subsequently divided into aliquots that were equilibrated with 4 different partial pressures of pCO_2 to allow determination of α and pK_{app} as described below. Equilibration of ruminal fluid was achieved with a humidified CO_2/N_2 gas mixture via the rotating flask method (Heisler, 1986) in a water bath at $38^\circ C$ for at least 60 min (Figure 1a). The $CO_2:N_2$ mixtures were generated using a gas mixing pump (Woesthoff 1M301/a-F, Woesthoff, Bochum, Germany). Partial pressure of CO_2 was calculated by multiplying the fraction of CO_2 in the gas mixture with ambient pressure corrected for humidity at $38^\circ C$ using the Magnus equation (Terblanche et al., 2008).

Determination of Total Carbonate Concentration $[CO_2]_{tot}$. Determination of α and pK_{app} according to the Astrup technique requires determination of

$[CO_2]_{tot}$, which is the sum of bicarbonate and dissolved CO_2 . For this purpose, a Foxbox CO_2 measuring system (Sable Systems, Las Vegas, NV) was equipped with a reaction vessel for the sample. An internal gas pump continuously flushed gas from the external vessel to an infrared CO_2 sensor. A long thin tube from the vessel to the outside served to keep the pressure within the system at ambient levels (see Figure 1b).

The measuring device was calibrated on a daily basis both before and after the measurements by injecting 0.5 mL of a $100 \text{ mmol}\cdot\text{L}^{-1}$ $NaHCO_3$ solution through an injection port into the reaction vessel. After acidification with a small amount of $1 \text{ mol}\cdot\text{L}^{-1}$ H_2SO_4 to $pH < 4$, the $[HCO_3^-]$ in the sample was protonated to form CO_2 gas (Cameron, 1971; Heisler, 1986), which was then stripped off the sample by the continuous flow of the carrier gas through the system and flushed to the Foxbox CO_2 sensor. On average, the coefficient of variation 4 consecutive measurements was 5.8%. The system was aerated with ambient air if CO_2 content exceeded 2% in the carrier gas.

For measurements, 0.5 mL of the equilibrated ruminal fluid sample was slowly aspirated from the flask (Figure 1a) using a gas-tight syringe. Care was taken to avoid formation of gas bubbles due to negative pressure. After injection into the reaction vessel and acidification ($1 \text{ mol}\cdot\text{L}^{-1}$ H_2SO_4), all $[HCO_3^-]$ in the sample was converted into gaseous CO_2 , which was driven off the solution by the carrier gas flowing through the system and measured with the CO_2 sensor of the Foxbox. Foxbox analog outputs were digitized with a resolution of 12 bits and transferred to a personal computer at $0.2 \text{ samples}\cdot\text{s}^{-1}$ and immediately analyzed (TurboLab software, MDZ Bühler & Partner, Münster, Germany). Steady state values of CO_2 were assumed to have been achieved if the difference between 2 consecutive averages of 60 s was less than 0.2% of the maximal range of the measurement. Afterward, residual fluid from the reaction vessel was sucked off and the measurement was repeated twice. If the difference between 3 of these iterations exceeded 5%, a fourth sample was analyzed.

Determination of the Solubility α of CO_2 . The total carbon dioxide concentration $[CO_2]_{tot}$ is given by the bicarbonate concentration in the sample $[HCO_3^-]$ and the partial pressure of CO_2 (pCO_2) according to

$$[CO_2]_{tot} = [HCO_3^-] + \alpha \cdot pCO_2. \quad [1]$$

For pH values < 4 , the $[HCO_3^-]$ in the sample is negligible, so that

$$\alpha \sim [CO_2]_{tot}^{HCO_3^- \text{ free}} / pCO_2 \quad [2]$$

for $\text{pH} < 4$. Accordingly, aliquots of ruminal fluid were acidified to $\text{pH} < 4$ before the measurement to drive out preexisting HCO_3^- . The acidified samples were then equilibrated with a known pCO_2 and $[\text{CO}_2]_{\text{tot}}^{\text{HCO}_3^- \text{ free}}$ measured using the Foxbox system as described above. Values for α were obtained for each individual sample of ruminal fluid.

Determination of pK_{app} of the Bicarbonate System. The apparent dissociation constant pK_{app} can be calculated from the Henderson–Hasselbalch equation defined as

$$\begin{aligned} \text{pH} &= \text{pK}_{\text{app}} + \log_{10} \frac{[\text{HCO}_3^-]}{\alpha \cdot \text{pCO}_2} \\ &= \text{pK}_{\text{app}} + \log_{10} \frac{[\text{CO}_2]_{\text{tot}} - \alpha \cdot \text{pCO}_2}{\alpha \cdot \text{pCO}_2}. \end{aligned} \quad [3]$$

Once α has been determined, the pK_{app} of each sample could be determined using this relationship by measuring the pH and the $[\text{CO}_2]_{\text{tot}}$ of the sample. The pK_{app} was obtained for each sample of ruminal fluid at each pCO_2 using equation [3]. Values of pK_{app} from the 4 ali-

Figure 1. Experimental setup used for measurements with the Astrup technique: (a) the partial pressure of CO_2 was set to defined values using a gas mixing pump. Equilibration to this partial pressure of CO_2 (pCO_2) occurred at 38°C for at least 60 min; the solution was stirred via rotation of the flask to produce a thin film of the fluid on the wall of the flask, maximizing the surface area for gas exchange (Heisler, 1986). (b) After equilibration, a sample was introduced into the reaction vessel of the measuring device and acidified to $\text{pH} < 4$ with H_2SO_4 to protonate the HCO_3^- in the sample. After dissociation and dehydration of H_2CO_3 , the resulting CO_2 was flushed out of the reaction vessel to a CO_2 sensor, driven by a continuous stream of carrier gas through the system. The system was aerated with ambient air if CO_2 content exceeded 2% in the carrier gas. The amount of CO_2 added per measurement was recorded by a personal computer (TurboLab Software, MDZ Bühler & Partner, Münster, Germany).

quotes were averaged to yield one mean value for each ruminal sample.

Determination of the Nonbicarbonate Buffer Capacity β_{NB} by Titration with CO_2 . When a solution is equilibrated with gaseous CO_2 , protons originating from the hydration and dissociation of CO_2 are not just in equilibrium with preexisting HCO_3^- , but are also bound by nonbicarbonate buffering anions (e.g., SCFA). This changes the concentration of the protonated complex ($\Delta [H^+ - Buffer^-]$), so that the HCO_3^- concentration will rise with the concentration of physically solved CO_2 ($[CO_2]$) according to

Within the physiological pH range of 5 to 8, the concentration of free protons ($[H^+]$) is very small and the $\Delta[HCO_3^-]$ that is formed from CO_2 essentially reflects the amount of protons buffered by the nonbicarbonate buffers. This allows an assessment of the buffer capacity of nonbicarbonate anions, β_{NB} , which can be determined from the change in pH (ΔpH) and the change in bicarbonate concentration ($\Delta [HCO_3^-]$) according to (Heisler, 1986):

$$\beta_{NB} = \frac{\Delta[OH^-]}{\Delta pH} = -\frac{\Delta[H^+]}{\Delta pH} = -\frac{\Delta[HCO_3^-]}{\Delta pH}. \quad [5]$$

To gain an estimate of β_{NB} , aliquots of the various samples were equilibrated with 4 different values of pCO_2 (ranging from 2.0 to 86.3 kPa) with subsequent determination of pH and $[CO_2]_{tot}$, from which $[HCO_3^-]$ could be determined using equation [1]. After plotting $[HCO_3^-]$ against the pH in a Davenport diagram (Davenport, 1974), the buffer capacity β_{NB} of the sample was determined from the slope of the linear regression (equation [5]).

Determination of α and pK_{app} and the Base Excess from Titration Curves at Differing pCO_2 . A second approach was used to validate the results obtained from the Astrup technique. The method uses the fact that the buffer capacity of various components of a multi-buffer system are additive (Heisler, 1986), so that the total buffer capacity β_{tot} of a system is given by the sum of the buffer capacity of all nonbicarbonate buffers ($\Sigma\beta_{NB}$) and the contribution of the bicarbonate system ($\beta_{bicarbonate}$) according to

$$\beta_{tot} = \Sigma\beta_{NB} + \beta_{bicarbonate}. \quad [6]$$

If β_{tot} of the solution is measured by simple titration over a range of pH values at 2 different pCO_2 , the re-

sulting curves can be subtracted from each other so that the nonbicarbonate buffers ($\Sigma\beta_{NB}$) cancel out of the resulting difference function

$$\begin{aligned} \Delta\beta_{tot} &= (\Sigma\beta_{NB} + \beta_{bicarbonate(1)}) - (\Sigma\beta_{NB} + \beta_{bicarbonate(2)}) \\ &= \beta_{bicarbonate(1)} - \beta_{bicarbonate(2)}. \end{aligned} \quad [7]$$

According to Heisler (1986),

$$\beta_{bicarbonate} = \frac{\partial[HCO_3^-]}{\partial pH} = \frac{1}{\log_{10}(e)} \cdot [HCO_3^-] = 2.302 \cdot [HCO_3^-]$$

and

$$[HCO_3^-] = \alpha \cdot pCO_2 \cdot 10^{(pH-pK)}.$$

We get $\Delta\beta_{tot}$ as a function of pH:

$$\Delta\beta_{tot}(pH) = 2.302 \cdot \alpha \cdot \Delta pCO_2 \cdot 10^{(pH-pK)}, \quad [8]$$

where ΔpCO_2 is the difference between the 2 partial pressures used in the measurements. If $\Delta\beta_{tot}(pH)$ is measured for several different pH, the resulting curve can be fitted to equation [8] to yield values for α and pK_{app} .

Experimental Procedure. For these measurements, aliquots of 20 mL of ruminal fluid were kept at 38°C and equilibrated with humidified gas containing either 100% CO_2 or carbogen gas (95% O_2 , 5% CO_2). After subtraction of the vapor pressure of H_2O at 38°C (~6.3 kPa), these conditions yield absolute pressure values of ~94.98 and ~4.75 kPa CO_2 , respectively. The HCl (1 mmol·L⁻¹) or NaOH (1 mmol·L⁻¹) were added in steps of 100 and 250 μ mol, respectively. The pH was measured for each titration step. Total buffer capacity was determined by dividing the ΔpH observed over 3 titration steps by the change in added acid or base ($\approx \Delta[HCl]$ or $\approx -\Delta[NaOH]$), correcting for dilution by the added amount. This value was assigned to the middle pH value of the respective triplet. In this manner, buffer capacity was determined over the entire pH range and 2 curves β_{tot} (pH, 94.98 kPa CO_2) and β_{tot} (pH, 4.75 kPa CO_2) were obtained.

After interpolation of the data along the pH axis in steps of $\Delta pH = 0.01$ and alignment along the pH axis, the 2 curves were subtracted to yield $\Delta\beta_{tot}$ (pH, ΔpCO_2), plotted over pH, and fitted to equation [8] yielding Henry's constant α and the pK_{app} of the bicarbonate system for the sample investigated (all using SigmaPlot for Windows, Version 11.0, Systat Software Inc., San Jose, CA). In the fitting process, an addition-

al variable was introduced into the fitting equation by subtraction from the measured pH to allow an estimate of the inaccuracy of the calibration of the pH meter, which was found to deviate from the theoretically expected value by a mean of -0.06 ± 0.02 pH units.

Determination of the Base Excess

In analogy to the concept established for systemic acid-base physiology, the concept of base excess was used. For the present purpose, the base excess (**BE**) was defined as the amount of acid required to reach pH values of 6.4 or 5.8 in the ruminal sample studied (referred to as $BE_{6.4}$ and $BE_{5.8}$, respectively). This value depends on both the initial pH of the sample and on the amount of (unprotonated) buffer in the pH range of the measurement. Accordingly, the base excess of a sample with a total concentration of $100 \text{ mmol}\cdot\text{L}^{-1}$ SCFA and an initial pH of 5 will be negative, as the addition of a base is required to adjust the pH to the desired level. Conversely, that of a sample with the same total concentration of SCFA but an initial pH of 7 will be positive. In ruminal samples, other buffers than SCFA can be expected to contribute.

In practice, the BE was determined from the titration data using 100% CO_2 -equilibrated ruminal fluid (or 94.98 kPa CO_2) via linear interpolation between the data points around pH 6.4 and 5.8, respectively. Here, as in all other parts of the study, results were corrected for the effect of dilution due to the addition of added acid or base.

Evaluation of Data and Statistics

Data were fitted to functions and compared statistically using SigmaPlot for Windows, Version 11.0, Systat Software Inc., and tested for significance using the Wilcoxon signed-rank test. Testing for correlation was performed using the Pearson product moment correlation (SigmaStat) for normally distributed data and the Spearman rank order correlation for not normally distributed data.

Results are presented as means $\pm$ standard error. The number of experimental animals is designated by N; the number of samples obtained as n.

RESULTS

Measurements of the pH in the Rumen, the Reticulum, and of Ruminal Samples in Vitro

The ruminal pH of all animals studied was measured both directly through the fistula and in samples directly after aspiration through the rumen. The pH of these aliquots was found to be significantly higher than that

measured directly in the rumen by an average of 0.26 ± 0.04 units ($P < 0.001$, $N/n = 18/32$). In the following, only the directly measured values will be discussed.

The ruminal pH of the hay/silage-fed animals was 6.55 ± 0.05 (6.47 ± 0.07 for animal h1, and 6.64 ± 0.09 for animal h2; $P = 0.078$, $n/n = 24/2$). In each animal, the pH measured before feeding was significantly higher than the value measured afterward ($P = 0.007$).

The ruminal pH measured in concentrate/silage-fed cows was significantly lower (5.52 ± 0.04 , $n/n = 8/8$; $P < 0.001$) than those of the hay/silage-fed animals. Mean reticular pH as measured by an electronic pH measuring bolus scattered around a mean that was 6.39 ± 0.10 , significantly higher than values measured in the rumen ($P < 0.001$, $n/n = 8/8$) and without any correlation to the pH measured directly in the rumen ($r^2 = 0.0004$). This discrepancy may reflect the higher concentration of salivary buffers in this part of the forestomachs.

Effect of $p\text{CO}_2$ on the pH and on the Bicarbonate Concentration $[\text{HCO}_3^-]$

When aliquots were equilibrated at defined $p\text{CO}_2$ [$100\% \text{ CO}_2$ ($\approx 94.98 \text{ kPa}$) and $5\% \text{ CO}_2$ ($\approx 4.75 \text{ kPa}$)], mean pH was 6.40 ± 0.03 at 95 kPa CO_2 and 7.63 ± 0.04 at 4.7 kPa CO_2 ($N/n = 2/12$) for the hay/silage-fed cows. These values differed significantly from each other and from those measured in samples from cows fed a concentrate/silage diet (both $P < 0.001$), where pH values of 5.58 ± 0.05 (95 kPa CO_2) and 5.85 ± 0.12 (4.7 CO_2) were observed ($P = 0.008$, $N/n = 8/8$).

To determine the effect of $p\text{CO}_2$ on $[\text{HCO}_3^-]$, aliquots of each ruminal sample were equilibrated with 4 different values of $p\text{CO}_2$ ranging randomly from 2 to 86 kPa. After determination of $[\text{CO}_2]_{\text{tot}}$ and α as described in the Materials and Methods section, $[\text{HCO}_3^-]$ could be calculated from equation [1]. The resulting pairs of pH and HCO_3^- are plotted in a Davenport diagram (Figure 2 for hay/silage-fed cows, Figure 3 for concentrate/silage-fed cows).

In 3 samples, HCO_3^- decreased unexpectedly with rising $p\text{CO}_2$. This may reflect either experimental error or release of nonvolatile H^+ ions that neutralize existing HCO_3^- in the sample (Heisler, 1986), possibly due to ongoing fermentation. Despite these 3 outliers, it can clearly be seen that rising levels of $p\text{CO}_2$ within the physiological range can be expected to have considerable effect on the measured pH of a sample.

Relationship Between Buffer Capacity and pH

In the pH range studied, over 99% of the protons formed after solution of CO_2 are bound to buffering

anions, and thus, the slopes of the linear regressions between each quadruplet of data points in the Davenport diagrams (Figures 2 and 3) give an estimate of the nonbicarbonate buffer capacity $\beta_{NB} = (\Delta[\text{HCO}_3^-] / -\Delta\text{pH})$ of each sample. Nonbicarbonate buffer capacity depended primarily on the pH at which the measurement was performed, which in turn primarily related to the pCO_2 . No influence of time after feeding ($P = 0.62$) or of the individual cow ($P = 0.62$) studied was detectable, although a tendency was found for higher buffer capacity in the concentrate/silage-fed animals ($P = 0.07$).

To assess the effect of pH on total buffer capacity over a range from ~ 3 to ~ 8 , equilibrated samples were titrated using HCl and NaOH (see Figure 4a). Both at a pCO_2 of 4.7 and 95 kPa, buffer capacity reached a local maximum at $\text{pH} \sim 4.8$, reflecting the significant contribution of SCFA to total buffering power. Because buffer capacities are additive, interpolation and subtrac-

tion of the curves at differing pCO_2 yield the isolated contribution of the bicarbonate system to total buffer capacity (Figure 4b). By fitting these difference curves to equation [8], it was possible to determine pK_{app} and α of the bicarbonate system for each ruminal sample (titration method).

Determination of α and pK_{app} of the Bicarbonate System via the Astrup and the Titration Technique

Using the Astrup technique with direct determination of $[\text{CO}_2]_{\text{tot}}$, the solubility of CO_2 (α) in ruminal fluid was found to be $0.244 \pm 0.01 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$ ($N/n = 10/31$). Solubility did not differ in the various feeding groups ($P = 0.43$), was not influenced by the time after feeding ($P = 0.9$), and showed no correlation to the SCFA concentration (Pearson coefficient $P > 0.05$).

Figure 2. Davenport diagram showing a plot of the pH measured against the HCO_3^- concentration for samples taken from 2 hay/silage-fed cows (h1, h2). Samples were divided into 4 aliquots and equilibrated with 4 different values of partial pressure of CO_2 (pCO_2). The pH and the $[\text{HCO}_3^-]$ of each of the 4 equilibrated samples were determined (Figure 1 and Materials and Methods section), plotted against each other, and fitted by linear regression. Because in the pH range studied, protons freed by the dissociation of CO_2 are almost completely absorbed by the nonbicarbonate buffers of the system, the absolute value of the slope of this regression reflects the nonbicarbonate buffer capacity, $\beta_{NB} \approx -\Delta[\text{HCO}_3^-] / \Delta\text{pH}$, of each ruminal sample. The curved lines in the figure represent isobars of 95, 66, 37, and 2 kPa of CO_2 and were calculated using equation [3] and the corresponding pK_{app} . The gray area corresponds to the physiological range of ruminal pCO_2 from 37 to 66 kPa.

Using curve fitting after titration as described above, a value of $\alpha = 0.223 \pm 0.003 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$ was obtained for the hay/silage-fed animals ($N/n = 2/24$) and $0.232 \pm 0.012 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$ for the concentrate/silage-fed animals ($N/n = 8/8$). No difference emerged between the feeding groups ($P = 0.7$) or versus the values for α measured with the Astrup technique ($P = 0.152$).

A mean dissociation constant pK_{app} of 6.21 ± 0.03 ($N/n = 10/32$) of all animals studied was found using the Astrup technique. Again, no significant difference emerged between hay/silage- and concentrate/silage-fed animals ($P = 0.3$), and no correlation was found between the pK_{app} and the feeding regimen ($P = 0.18$), the time after feeding ($P = 0.6$), or the SCFA concentration in the sample ($P = 0.6$). The values obtained for pK_{app} using the titration method (6.05 ± 0.01 for hay/silage-fed animals and 5.97 ± 0.05 for concentrate/silage-fed animals) were slightly, but significantly, lower than those obtained with the Astrup technique ($P < 0.001$) but again did not differ among each other ($P = 0.7$).

In conjunction and using 2 independent methods, values of $\alpha = 0.234 \pm 0.005 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$ and pK_{app}

$= 6.11 \pm 0.02$; $n/n = 31/10$) were obtained for ruminal fluid, essentially identical to those reported for plasma (Andersen, 1962; Segel, 1976).

Determination of the SCFA Concentration from the Titration Curves

In an attempt to estimate the SCFA concentration ($[\text{SCFA}]_{\text{fit}}$) from the buffer curves, the titration curves were fitted by the equation

$$\beta_{\text{tot}}(\text{pH}) = 2.302 \cdot \alpha \cdot \text{pCO}_2 \cdot 10^{(\text{pH}-\text{d}-\text{pK}_{\text{app}})} + 2.302 \cdot \frac{[\text{SCFA}]_{\text{fit}}}{2 + 10^{(\text{pH}-\text{d}-\text{pK})} + 10^{(\text{pK}-(\text{pH}-\text{d}))}}, \quad [9]$$

where $\text{pK}_{\text{SCFA}} \approx 4.76$ and d is the inaccuracy of the calibration of the pH meter.

For the hay/silage-fed animals, a mean value for the variable $[\text{SCFA}]_{\text{fit}}$ in this equation of $81 \pm 4 \text{ mmol} \cdot \text{L}^{-1}$ was obtained. This value is in surprisingly good agreement with the mean of the total concentration of SCFA, $[\text{SCFA}]_{\text{measured}}$, as determined by gas chromatography ($80 \pm 2 \text{ mmol} \cdot \text{L}^{-1}$, hay/silage-fed cows).

Figure 3. Davenport diagram showing the HCO_3^- concentration over the measured pH of ruminal samples taken from 8 concentrate/silage-fed cows (designated c1 to c8). For explanation, see Figure 2 and text. pCO_2 = partial pressure of CO_2 .

Figure 4. (a) Buffer capacity obtained from one ruminal sample (h1, d 15, 0700 h) after titration at 2 values of partial pressure of CO_2 [partial pressure of CO_2 (pCO_2); 5% $\text{CO}_2 \approx 4.75$ kPa and 100% $\text{CO}_2 \approx 94.98$ kPa; $\Delta\text{pCO}_2 = 90.23$ kPa; 38°C]. The dashed line was calculated using the concentrations of individual short-chain fatty acids (SCFA) as measured by gas chromatography according to $\beta_{\text{NB}} = 2.302 \cdot [\text{acetate}] / (2 + 10^{(\text{pH} - 4.76)} + 10^{(4.76 - \text{pH})}) + 2.302 \cdot [\text{propionate}] / (2 + 10^{(\text{pH} - 4.88)} + 10^{(4.88 - \text{pH})}) + 2.302 \cdot [\text{butyrate}] / (2 + 10^{(\text{pH} - 4.82)} + 10^{(4.82 - \text{pH})})$. The dash-dotted and dotted lines reflect the contribution of bicarbonate to total buffer capacity and were calculated from $\beta_{\text{bicarbonate}} = 2.302 \cdot \alpha \cdot \text{pCO}_2 \cdot 10^{(\text{pH} - \text{pK}_{\text{app}})}$ for values of pCO_2 of 100 and 5%, respectively, and $\text{pK} = 6.1$ and $\alpha = 0.226$ $\text{mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$. The solid line represents the sum $\beta_{\text{tot}}(\text{pH}) = \beta_{\text{bicarbonate}} + \beta_{\text{NB}}$ of both curves and reflects the contribution of SCFA and bicarbonate to total buffer capacity. The data could also be fitted without prior knowledge of SCFA concentration, yielding a slightly better fit and an orientation for the total SCFA in the sample ($[\text{SCFA}]_{\text{fit}}$) (see text for details). (b) The figure shows the difference $\Delta\beta_{\text{tot}}(\text{pH})$ between the buffer capacity at 4.75 and 94.98 kPa CO_2 at each value of pH after interpolation of the individual curves of all hay/silage-fed animals. The buffer capacity of the nonbicarbonate buffers is cancelled out of the equation, and accordingly, data could be fitted via $\Delta\beta_{\text{tot}} = \beta_{\text{bicarbonate}} = 2.302 \cdot \alpha \cdot \Delta\text{pCO}_2 \cdot 10^{(\text{pH} - \text{pK}_{\text{app}})}$. For the hay/silage-fed animals, the approach yielded $\alpha = 0.223 \pm 0.003$ $\text{mmol} \cdot \text{L}^{-1} \cdot \text{kPa}^{-1}$, $\text{pK}_{\text{app}} = 6.049 \pm 0.009$, and a mean calibration error of the pH electrode $d = 0.041 \pm 0.007$ pH units.

Regression analysis of the fitting results over the measured SCFA values yielded $[\text{SCFA}]_{\text{fit}} = -5.7$ $\text{mmol} \cdot \text{L}^{-1} + 1.07 \cdot [\text{SCFA}]_{\text{measured}}$ ($R^2 = 0.60$). In the concentrate/silage-fed animals, $[\text{SCFA}]_{\text{measured}}$ was 89 ± 4 $\text{mmol} \cdot \text{L}^{-1}$, which was significantly lower than that determined via titration of $[\text{SCFA}]_{\text{fit}} = 125 \pm 7$ $\text{mmol} \cdot \text{L}^{-1}$ ($P < 0.001$). In its present form, the titration method clearly cannot be considered a suitable alternative for the estimation of the SCFA concentration in ruminal fluid.

Base Excess

The amount of acid necessary to adjust the samples to a pH of 6.4 or 5.8 was determined for samples

equilibrated with 100% CO_2 (94.98 kPa) by interpolation from the titration curves. In the hay/silage-fed animals, a mean of 0.5 ± 3 $\text{mmol} \cdot \text{L}^{-1}$ ($\text{BE}_{(6.4)}$) or 39.2 ± 2.8 ($\text{BE}_{(5.8)}$) of HCl had to be added, reflecting the amount of acid necessary to adjust sample pH to 6.4 or 5.8, respectively. Both values were in close agreement with those obtained from equations derived from Henderson–Hasselbalch theory (see Appendix), again suggesting that SCFA is the primary nonbicarbonate buffer present in this group of animals. Accordingly, the correlation between BE and ruminal pH was acceptable ($\text{BE}_{(6.4)}$: $R^2 = 0.72$, $\text{BE}_{(5.8)}$: $R^2 = 0.63$), as was the correlation between BE and SCFA concentration (Pearson product moment $P < 0.01$ for both $\text{BE}_{(6.4)}$ and $\text{BE}_{(5.8)}$).

In the concentrate/silage-fed animals, the starting pH was below 6.4 and NaOH had to be added, resulting in a mean $BE_{(6.4)}$ of $-93 \pm 18.5 \text{ mmol}\cdot\text{L}^{-1}$ and $BE_{(5.8)}$ of $-22.2 \pm 13.8 \text{ mmol}\cdot\text{L}^{-1}$ for samples gassed with 100% CO_2 ($P < 0.0001$ versus hay/silage). The BE showed no correlation to pH as measured directly in the rumen ($BE_{(6.4)}$: $R^2 = 0.004$, $BE_{(5.8)}$: $R^2 = 0.002$) or in the reticulorumen by a pH-sensitive probe ($BE_{(6.4)}$: $R^2 = 0.06$, $BE_{(5.8)}$: $R^2 = 0.013$). In this group of animals, $BE_{(5.8)}$ was found to scatter strongly, with values measured that ranged from -50 to $-120 \text{ mmol}\cdot\text{L}^{-1}$, although corresponding pH values were roughly the same (between 5.5 and 5.7). In the animals with a highly negative base excess, buffering anions were apparently highly protonated so that much base was necessary before the pH could be shifted to the desired range. Note that in the corresponding animals, these protonated buffers have to be absorbed from the rumen with potential negative effect on intraepithelial pH homeostasis.

In contrast to what was observed in the hay/silage fed animals, no correlation could be found between the total or individual SCFA concentrations as measured by gas chromatography and the BE in the concentrate/silage-fed animals. This is not surprising because gas chromatography does not distinguish between the protonated and the unprotonated form of SCFA. In the pH range in the concentrate/silage group (5.7 to 5.3), the percentage of protonated SCFA can be expected to vary from 11 to 24%. In addition, other buffers may have contributed to total buffering power in this group of animals (see Appendix). Notably, the protons bound by these buffers were released upon titration to higher values of pH and can be expected to burden the acid-base balance of cells within the ruminal epithelium during the absorption process. In practical terms, measuring base excess may be the simplest way to assess the contribution of multiple buffers to the acid load of the epithelium and estimate the total quantity of protons bound.

DISCUSSION

Understanding ruminal buffering is not possible if no reliable values for the buffer constants of the bicarbonate system are available. In this study, 2 independent methods were used to measure the solubility of CO_2 (Henry's constant α) and for the pK_{app} of H_2CO_3 in ruminal fluid at 38°C . The values found [$\alpha = 0.234 \pm 0.005 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{kPa}^{-1}$, $pK_{\text{app}} = 6.11 \pm 0.02$; ($n/n = 31/10$)] were essentially identical to those established for either blood plasma or isotonic salt solution at 37°C (Andersen, 1962; Segel, 1976). Although this result is hardly surprising and has been previously postulated

(Kohn and Dunlap, 1998), we are not aware of any study where this has been stringently shown.

Our study further clearly shows that spurious changes in $p\text{CO}_2$ can significantly compromise measurements of ruminal pH. We demonstrate that a more rigorous approach with equilibration of ruminal samples to a standard $p\text{CO}_2$ and subsequent determination of base excess is technically feasible and may prove useful in the diagnosis of disturbances of ruminal acid-base homeostasis. Furthermore, we introduce a new simple method for the determination of α and pK of the bicarbonate system that has not, to our knowledge, been described before. This method should be generally helpful for estimating α and pK in physiological multi-buffer solutions. Finally, in the Appendix, we provide equations suitable for estimating the response of a mixed SCFA/ HCO_3^- buffer solution to changes in various parameters such as $p\text{CO}_2$ and HCO_3^- .

Conversion of HCO_3^- to CO_2 in Ruminal Fluid

Although the principles underlying the conclusions of this study are hardly new, considerable debate has occurred concerning the extent to which salivary HCO_3^- is converted to CO_2 in the rumen, with implications both for understanding ruminal buffering and the driving forces relevant for transport physiology. Our direct measurements of the $[\text{HCO}_3^-]$ concentration in ruminal fluid at different values of pH and $p\text{CO}_2$ clearly show that the standard constants for bicarbonate buffering apply and that the classical isohydric principle fully holds for ruminal fluid (Nguyen et al., 2009). This principle states that the overall pH determines the relationship between the concentrations of each individual acid and base pair in a multi-buffer system. The HCO_3^- concentration of a sample is thus determined by pH and the $p\text{CO}_2$ as given by the classical Henderson-Hasselbalch equation.

In this context, it is important to realize that because $p\text{CO}_2$ is maintained at near constant levels due to eructation and absorption of CO_2 , the rumen has to be considered an open buffer system. This concept is well known from systemic acid-base physiology, where the bicarbonate system buffers plasma to pH 7.4, far from the pK of 6.1. The pH falls with rising values of $p\text{CO}_2$ and accordingly, hyperventilation with removal of CO_2 from the body via the lungs can correct metabolic acidosis, whereas clinically, infusions of NaHCO_3 shift pH back to the neutral range. Likewise, in the rumen, additional HCO_3^- (e.g., via influx of saliva or transport across the ruminal wall) buffers the system to values that are much more alkalic than the pK of the bicarbonate system (Kohn and Dunlap, 1998). Cor-

responding shifts in the ratio of acetate to acetic acid will follow according to

$$\begin{aligned} \text{pH} &= \text{pK}_{\text{H}_2\text{CO}_3} + \log_{10} \left(\frac{[\text{HCO}_3^-]}{\alpha \cdot \text{pCO}_2} \right) \\ &= \text{pK}_{\text{acetic acid}} + \log_{10} \left(\frac{[\text{acetate}^-]}{[\text{acetic acid}]} \right). \end{aligned} \quad [10]$$

Consequences for Transport Physiology

Using equation [10] and the constants determined in this study, it is possible to calculate the concentration of $[\text{HCO}_3^-]$ in the rumen (or any other organ of the fermentative gut) for any given value of pH and pCO_2 , thus allowing an estimate of the driving forces for transport processes.

In the rumen, a large albeit variable fraction of the protons produced in the fermentational process are neutralized by salivary NaHCO_3 , forming H_2O and CO_2 , whereas SCFA anions are absorbed with Na^+ (or K^+ released from forage) in a process that minimizes the acid load of the epithelium (Aschenbach et al., 2011). At a total concentration of $100 \text{ mmol} \cdot \text{L}^{-1}$ SCFA and pH 6.1, the concentration of the protonated form (H-SCFA) is less than $\sim 5 \text{ mmol} \cdot \text{L}^{-1}$, so that the rest of the SCFA anions (or $\sim 95 \text{ mmol} \cdot \text{L}^{-1}$) are coupled to strong cations such as Na^+ and K^+ , reflecting the concentrations found via direct measurement (Majak and Hall, 1989).

Due to the shortage of protons, net transport of SCFA across the rumen and other fermentative organs can thus hardly proceed via lipid diffusion alone. Current models of SCFA transport involve an apical anion exchanger with secretion of buffering HCO_3^- (Hume et al., 1993; Kramer et al., 1996; Aschenbach et al., 2009; Dengler et al., 2014). At a pH of 6.1 and a pCO_2 of 60 kPa, $[\text{HCO}_3^-]$ will drop to values under $15 \text{ mmol} \cdot \text{L}^{-1}$ (see Figures 2 and 3), contributing to the driving force for the uptake of SCFA anions via SCFA/ HCO_3^- exchange. At high ruminal pH, a high intracellular $[\text{HCO}_3^-]_i$ (maintained by apical influx of CO_2 , basolateral influx of HCO_3^- , efflux of protons to either side, or a combination of these) and a rapid basolateral extrusion of SCFA can maintain the driving force necessary for the uptake of individual SCFA in exchange for HCO_3^- .

Numerous studies demonstrate the involvement of apical sodium-proton exchange (NHE3) in restoring cytosolic pH, which is challenged both by influx of protons and by loss of HCO_3^- (Gäbel et al., 1991; Rabbani et al., 2011). This mechanism supplies protons to the apical side, protonating SCFA for influx

via diffusive mechanisms and maintaining the apical HCO_3^- gradient for anion exchange. In an epithelium in which intracellular pH is maintained by an apical NHE (Rabbani et al., 2011), a net uptake of SCFA $^-$ with Na^+ will follow. Basolateral efflux of SCFA anions could proceed through a large anion channel (Stumpff et al., 2009; Georgi et al., 2014), driven by the gradient generated by basolateral potassium channels and the Na^+/K^+ -ATPase.

In situations where salivary secretion is insufficient, an increasing amount of protons will have to be transported across the epithelium and eliminated basolaterally, involving various transport proteins such as basolateral NHE1 (Rabbani et al., 2011), NaHCO_3^- co-transport (Huhn et al., 2003), and an anion exchanger that extrudes SCFA $^-$ anions in exchange for HCO_3^- (Dengler et al., 2014).

Base Excess

In the evaluation of systemic acid-base balance, clinicians have long determined 3 parameters: pH, actual pCO_2 , and BE, which is equivalent to the amount of acid required to return the pH of a blood sample to a normal value (7.4) under standard conditions (37°C , $\text{pCO}_2 = 5.33 \text{ kPa}$; Jorgensen and Astrup, 1957). For ruminal samples, standard pCO_2 might be set to $\sim 60 \text{ kPa}$, although from our current perspective, utilization of 100% CO_2 , which is available at low cost in most laboratories, may represent an alternative more feasible in practical and economic terms as a standard. In this study, pH values both above (6.4) and on the threshold to subacute ruminal acidosis (5.8) were chosen as standards for determination of BE, although a higher pH value reflecting cytosolic pH might be an alternative worth considering.

Determination of the BE of a sample makes it possible to distinguish between unprotonated and protonated buffers. A negative BE thus reflects the amount of protons that will have to be absorbed across the ruminal wall and buffered by cellular mechanisms to prevent epithelial damage. Theoretically at least, the BE should be useful as a diagnostic tool particularly at low pH when the buffering capacity of SCFA rises. In this situation, large changes in the production of acid will have little effect on ruminal pH, but a major effect on the total amount of protons that enter the cytosol and challenge epithelial homeostasis (Enemark, 2008; Stumpff et al., 2009). Ultimately, clinical trials will have to show whether determination of the BE will yield greater diagnostic accuracy in determining what animals are likely to develop clinical symptoms than classical approaches.

Consequences for the Evaluation of Ruminal Acid-Base Balance

Consequences for many clinical situations abound. Attempts to reduce the levels of methane in the rumen by defaunation might result in higher levels of $p\text{CO}_2$, and thus reduce ruminal pH. Simultaneously, ruminal buffer capacity will increase (equation [9]). The clinical implications of this are hard to predict theoretically, but investigations are clearly necessary.

Most importantly, any spurious drop in the $p\text{CO}_2$ of samples of ruminal fluid, as observed when collecting samples from the rumen via an aspirating sampling device in this study and others (Enemark, 2008), can be expected to significantly affect the pH that is subsequently measured. Such variation in the $p\text{CO}_2$ may well underlie a significant part of the variability seen in studies attempting to correlate ruminal pH with clinical symptoms of ruminal acidosis.

In conclusion, the effect of changes in $p\text{CO}_2$, whether due to sampling technique or escape of CO_2 through a fistula opening or other factors, have to be considered when studying ruminal acidosis. An attempt to introduce a generally accepted protocol with fixed levels of temperature, $p\text{CO}_2$, and determination of BE for measuring the acid-base status of ruminal samples appears overdue.

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APPENDIX

Buffer Calculations for Solutions Containing SCFA and Bicarbonate

To understand ruminal buffering, intuition will frequently not be enough and it is useful to have a mathematical model that simulates how an ideal buffer solution containing carbon dioxide, bicarbonate, and SCFA will respond if one of the parameters is changed. For instance, the practitioner will wish to estimate by how much the pH of ruminal fluid can be expected to change if the pCO₂ changes (e.g., after aspiration of a sample using negative pressure), or in what way addi-

tions of NaHCO₃ (e.g., via saliva) will affect ruminal pH. Furthermore, it may be important to estimate the total amount of protons in bound and unbound form in the solution that have to be buffered or transported across the ruminal epithelium. Such calculations are possible but not entirely trivial. For the interested reader, the relevant equations will therefore be derived in this Appendix. A convenient spreadsheet for such calculations can be obtained from the authors upon request.

It will be shown in what follows that 3 independent parameters reflecting the amount of protons, the bicarbonate buffer, and the nonbicarbonate buffer are required to define the system and calculate the changes that occur following an intervention.

Calculating $[H^+]_{init}$, $[CO_2]_{tot}^{init}$ and $[strong\ cation^+]_{tot}$ from the Initial Parameters

We shall consider a buffer solution (e.g., ruminal fluid) with the following initial parameters:

total concentration of SCFA:

$$[SCFA]_{tot}^{init} = [HSCFA]_{init} + [SCFA^-]_{init} = 100\text{ mmol} \cdot L^{-1},$$

initial pH: $pH_{init} = 6.4$,

initial partial pressure of CO₂:

$$pCO_2^{init} = 70\text{ kPa},$$

where [SCFA]_{tot} designates the sum of the undissociated form ([HSCFA]) and the dissociated, anionic form ([SCFA[−]]). The relevant constants will be set as follows: $\alpha = 0.234\text{ mmol} \cdot L^{-1} \cdot kPa^{-1}$ (Henry's constant as determined in this study), $pK_{CO_2} = 6.11$ (dissociation constant of bicarbonate), and $pK_{SCFA} \approx 4.8$ (mean dissociation constant of the SCFA).

The initial concentrations of [H₂CO₃], [HCO₃[−]], and the total concentration of carbon dioxide, [CO₂]_{tot}, are given by Henry's law and the Henderson–Hasselbalch equation:

$$[H_2CO_3]_{init} = \alpha \cdot pCO_2^{init} = 16.38\text{ mmol} \cdot L^{-1}, \quad [A1]$$

$$[HCO_3^-]_{init} = \alpha \cdot pCO_2^{init} \cdot 10^{(pH - pK_{CO_2})} = 31.94\text{ mmol} \cdot L^{-1}, \quad [A2]$$

$$[CO_2]_{tot}^{init} = \alpha \cdot pCO_2^{init} \cdot (1 + 10^{(pH_{init} - pK_{CO_2})}) = 48.32\text{ mmol} \cdot L^{-1}, \quad [A3]$$

Likewise, the initial concentrations of undissociated and dissociated SCFA can be calculated from

$$\begin{aligned} \text{pH}_{\text{init}} &= \text{pK}_{\text{SCFA}} + \log_{10} \left(\frac{[\text{SCFA}^-]_{\text{init}}}{[\text{HSCFA}]_{\text{init}}} \right) = \\ &\text{pK}_{\text{SCFA}} + \log_{10} \left(\frac{[\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{init}}}{[\text{HSCFA}]_{\text{init}}} \right) \Leftrightarrow \\ &[\text{HSCFA}]_{\text{init}} = \frac{[\text{SCFA}]_{\text{tot}}}{(1 + 10^{(\text{pH} - \text{pK}_{\text{SCFA}})})} = 2.45 \text{ mmol} \cdot \text{L}^{-1}, \end{aligned} \quad [\text{A4}]$$

$$\begin{aligned} [\text{SCFA}^-]_{\text{init}} &= [\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{init}} \\ &= \frac{10^{(\text{pH} - \text{pK}_{\text{SCFA}})} \cdot [\text{SCFA}]_{\text{tot}}}{(1 + 10^{(\text{pH} - \text{pK}_{\text{SCFA}})})} = 97.55 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned} \quad [\text{A5}]$$

For $\text{pH} > 4$, the concentration of free $[\text{H}^+] = 10^{-\text{pH}}$ is negligible, so that the total initial concentration of protons $[\text{H}^+]_{\text{tot}}^{\text{init}}$ is given by

$$\begin{aligned} [\text{H}^+]_{\text{tot}}^{\text{init}} &= [\text{H}_2\text{CO}_3]_{\text{init}} + [\text{HSCFA}]_{\text{init}} + 10^{-\text{pH}_{\text{init}}} \\ &\cong \alpha \cdot \text{pCO}_2^{\text{init}} + \frac{[\text{SCFA}]_{\text{tot}}}{(1 + 10^{(\text{pH} - \text{pK}_{\text{SCFA}})})} = 18.83 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned} \quad [\text{A6}]$$

For reasons of electroneutrality, the total concentration of strong cations [that is, cations other than free H^+ (e.g., Na^+ , K^+)] is given by the sum of the free anions in the system:

$$\begin{aligned} [\text{strong cation}^+]_{\text{tot}} &= [\text{HCO}_3^-] + [\text{SCFA}^-] \\ &= [\text{CO}_2]_{\text{tot}} + [\text{SCFA}]_{\text{tot}} - [\text{H}^+]_{\text{tot}} = 129.5 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned} \quad [\text{A7}]$$

Conserved Quantities of the Buffer System

Before any calculations are possible, it is important to identify what quantities of the system are conserved when the pH or the pCO_2 change or both change.

The first conserved quantity of the system is the total amount of strong cations as given by equation [A7]. For instance, addition of 20 mmol of NaHCO_3 to 1 L of the mixed buffer solution above will have a complex effect on the pH and on the concentration of $[\text{HCO}_3^-]$ in the system, with both changing in response to changes in the pCO_2 . However, regardless of pH and pCO_2 , the total amount of strong cations in the solution from above can easily be calculated according to

$$\begin{aligned} [\text{strong cation}^+]_{\text{tot}}^{\text{end}} &= [\text{strong cation}^+]_{\text{tot}}^{\text{init}} + \Delta[\text{strong cation}^+]_{\text{add}} \\ &= 129.5 \text{ mmol} \cdot \text{L}^{-1} + 20 \text{ mmol} \cdot \text{L}^{-1} = 149.5 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned}$$

In this equation, $\Delta[\text{strong cation}^+]_{\text{add}}$ designates the amount of cations added during the intervention, whereas the initial and final concentrations are designated with the corresponding superscripts.

Furthermore, the total concentration of SCFA, SCFA_{tot} , does not change with pH and pCO_2 , although shifts in the ratio of the protonated and unprotonated form away from the initial values $[\text{HSCFA}]_{\text{init}}$ and $[\text{SCFA}^-]_{\text{init}}$ will, of course, occur.

$$\begin{aligned} [\text{SCFA}]_{\text{tot}}^{\text{init}} &= [\text{HSCFA}]_{\text{init}} + [\text{SCFA}^-]_{\text{init}} \\ &= [\text{SCFA}]_{\text{tot}}^{\text{end}} = [\text{SCFA}]_{\text{tot}} = \text{constant}. \end{aligned} \quad [\text{A8}]$$

Given that both the total concentration of strong cations and the total concentration of SCFA are conserved, equation [A7] yields a third conserved quantity of the system, namely the bicarbonate excess, $[\text{BCE}]_{\text{tot}}$, of the system, defined as the difference between $[\text{CO}_2]_{\text{tot}}$ and $[\text{H}^+]_{\text{tot}}$:

$$\begin{aligned} [\text{BCE}]_{\text{tot}} &= [\text{CO}_2]_{\text{tot}} - [\text{H}^+]_{\text{tot}} = [\text{strong cation}^+]_{\text{tot}} \\ &- [\text{SCFA}]_{\text{tot}} = \text{constant} = 29.49 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned} \quad [\text{A9}]$$

Using $[\text{CO}_2]_{\text{tot}} = [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-]$ and $[\text{H}^+]_{\text{tot}} = [\text{HSCFA}] + [\text{H}_2\text{CO}_3]$, it follows that the difference between $[\text{HCO}_3^-]$ and $[\text{HSCFA}]$ is also conserved:

$$\begin{aligned} [\text{BCE}]_{\text{tot}} &= [\text{H}_2\text{CO}_3]_{\text{init}} + [\text{HCO}_3^-]_{\text{init}} - ([\text{HSCFA}]_{\text{init}} \\ &+ [\text{H}_2\text{CO}_3]_{\text{init}}) = [\text{HCO}_3^-]_{\text{init}} - [\text{HSCFA}]_{\text{init}} \\ &= [\text{HCO}_3^-]_{\text{end}} - [\text{HSCFA}]_{\text{end}} = [\text{BCE}]_{\text{tot}}. \end{aligned} \quad [\text{A10}]$$

Equation [A10] also follows directly from the fact that in a mixed buffer system, H^+ ions generated from hydration and dissociation of CO_2 are buffered by nonbicarbonate buffers so that the increase in the protonated form of the nonbicarbonate buffer ($\Delta[\text{HSCFA}]$) is equal to the change in bicarbonate ($\Delta[\text{HCO}_3^-]$) (Heisler, 1986).

A final conserved quantity can be obtained using the isohydric principle, according to which the pH of a mix of buffers can be calculated from each buffer pair at any time point using the Henderson–Hasselbalch equation:

$$\begin{aligned}\text{pH} &= \text{pK}_{\text{SCFA}} + \log_{10}([\text{SCFA}^-]/[\text{HSCFA}]) \\ &= \text{pK}_{\text{CO}_2} + \log_{10}([\text{HCO}_3^-]/[\text{H}_2\text{CO}_3]).\end{aligned}$$

Using

$$C = \log_{10}(a) - \log_{10}(b) = \log_{10}(a/b) \Leftrightarrow 10^C = a/b,$$

we get

$$\frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \cdot \frac{[\text{HSCFA}]}{[\text{SCFA}^-]} = 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} = \text{constant} = 0.049. \quad [\text{A11}]$$

Equations for the Open Buffer System

We now wish to know how the open system changes with a variation of the initial parameters (e.g., when the pCO_2 drops). This might be the case if a ruminal sample were aspired using a syringe, or if the partial pressure of methane in the rumen rises, displacing CO_2 .

Let $[\text{HCO}_3^-]_{\text{end}}$ and $[\text{HSCFA}]_{\text{end}}$ be the concentrations of bicarbonate and of protonated SCFA after the drop in pCO_2 .

Using

$$[\text{H}_2\text{CO}_3]_{\text{end}} = \alpha \cdot \text{pCO}_2,$$

(Henry's law)

$$[\text{HCO}_3^-]_{\text{end}} = [\text{BCE}]_{\text{tot}} + [\text{HSCFA}]_{\text{end}},$$

(from equation [A10])

$$[\text{SCFA}^-]_{\text{end}} = [\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{end}},$$

(from equation [A8])

and using [A11], we get

$$\begin{aligned}10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} &= \frac{[\text{HCO}_3^-]_{\text{end}}}{[\text{H}_2\text{CO}_3]_{\text{end}}} \cdot \frac{[\text{HSCFA}]_{\text{end}}}{[\text{SCFA}^-]_{\text{end}}} \\ &= \frac{([\text{BCE}]_{\text{tot}} + [\text{HSCFA}]_{\text{end}})}{\alpha \cdot \text{pCO}_2} \cdot \frac{[\text{HSCFA}]_{\text{end}}}{([\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{end}})} \Leftrightarrow \\ &[\text{HSCFA}]_{\text{end}}^2 + ([\text{BCE}]_{\text{tot}} + \alpha \cdot \text{pCO}_2^{\text{end}} \cdot 10^{(\text{pK}_{\text{SCFA}} + \text{pK}_{\text{CO}_2})}) \\ &\times [\text{HSCFA}]_{\text{end}} + \alpha \cdot \text{pCO}_2^{\text{end}} \cdot 10^{(\text{pK}_{\text{SCFA}} + \text{pK}_{\text{CO}_2})} \cdot [\text{SCFA}]_{\text{tot}} = 0. \quad [\text{A12}]\end{aligned}$$

This quadratic equation can be solved in the usual manner, yielding

$$[\text{HSCFA}]_{\text{end}} = -\frac{([\text{BCE}]_{\text{tot}} + \alpha \cdot \text{pCO}_2^{\text{end}} \cdot 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})})}{2} \pm \sqrt{\frac{([\text{BCE}]_{\text{tot}} + \alpha \cdot \text{pCO}_2^{\text{end}} \cdot 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})})^2}{4} + \alpha \cdot \text{pCO}_2^{\text{end}} \cdot 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} \cdot [\text{SCFA}]_{\text{tot}}}. \quad [\text{A13}]$$

With the initial parameters from our example above,

$$\begin{aligned}\text{pH}_{\text{init}} &= 6.4, \text{pCO}_2^{\text{init}} = 70 \text{ kPa}, [\text{SCFA}]_{\text{tot}} = 100 \text{ mmol} \cdot \text{L}^{-1} \\ \Rightarrow [\text{BCE}]_{\text{tot}} &= 29.49 \text{ mmol} \cdot \text{L}^{-1}, [\text{H}^+]_{\text{tot}}^{\text{init}} = 18.83 \text{ mmol} \cdot \text{L}^{-1} \quad [\text{A14}]\end{aligned}$$

and assuming that the pCO_2 drops to

$$\text{pCO}_2^{\text{end}} = 50 \text{ kPa},$$

we obtain

$$[\text{HSCFA}]_{\text{end}} = 1.80 \text{ mmol} \cdot \text{L}^{-1},$$

(from equation [A13])

$$[\text{H}_2\text{CO}_3]_{\text{end}} = \alpha \cdot \text{pCO}_2^{\text{end}} = 11.70 \text{ mmol} \cdot \text{L}^{-1},$$

(Henry's law)

$$[\text{SCFA}^-]_{\text{end}} = [\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{end}} = 98.20 \text{ mmol} \cdot \text{L}^{-1}, \quad [\text{A15}]$$

$$[\text{HCO}_3^-]_{\text{end}} = [\text{BCE}]_{\text{tot}} + [\text{HSCFA}]_{\text{end}} = 31.29 \text{ mmol} \cdot \text{L}^{-1}, \quad [\text{A16}]$$

$$\begin{aligned}\text{pH}_{\text{end}} &= \text{pK}_{\text{SCFA}} \\ &+ \log_{10}([\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{end}})/[\text{HSCFA}]_{\text{end}} = 6.54, \quad [\text{A17}]\end{aligned}$$

$$[\text{H}^+]_{\text{tot}}^{\text{end}} = [\text{HSCFA}]_{\text{end}} + [\text{H}_2\text{CO}_3]_{\text{end}} = 16.44 \text{ mmol} \cdot \text{L}^{-1}. \quad [\text{A18}]$$

Note that in a real buffer solution, pH values will be slightly more acidic ($\Delta\text{pH} \sim 0.1$ to 0.2) than given by these equations due to the fact that the activity constant of the unprotonated SCFA anion is significantly lower than that of the protonated form and changes

with the concentration of cations in a complex manner (Mortimer, 2008).

Equations for the Closed Buffer System

In a closed system, no CO₂ can escape. Accordingly,

$$\begin{aligned} [\text{CO}_2]_{\text{tot}}^{\text{init}} &= [\text{H}_2\text{CO}_3]_{\text{init}} + [\text{HCO}_3^-]_{\text{init}} \\ &= [\text{H}_2\text{CO}_3]_{\text{end}} + [\text{HCO}_3^-]_{\text{end}} = [\text{CO}_2]_{\text{tot}}^{\text{end}} = \text{constant}. \end{aligned} \quad [\text{A19}]$$

Again, bicarbonate excess ([BCE]_{tot}) is conserved (equation [A19]):

$$\begin{aligned} [\text{CO}_2]_{\text{tot}}^{\text{init}} - [\text{H}^+]_{\text{tot}}^{\text{init}} &= [\text{CO}_2]_{\text{tot}}^{\text{end}} - [\text{H}^+]_{\text{tot}}^{\text{end}} \\ &= [\text{BCE}]_{\text{tot}} = \text{constant}. \end{aligned}$$

This means that because [CO₂]_{tot} is conserved, the total concentration of protons is also conserved:

$$[\text{H}^+]_{\text{tot}}^{\text{init}} = [\text{H}^+]_{\text{tot}}^{\text{end}} = \text{constant}. \quad [\text{A20}]$$

Furthermore, the isohydric principle holds (equation [A11]), so that with equation [A10], we get

$$\begin{aligned} 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} &= \frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \cdot \frac{[\text{HSCFA}]}{[\text{SCFA}^-]} \\ &= \frac{([\text{BCE}]_{\text{tot}} + [\text{HSCFA}]_{\text{end}})}{([\text{H}^+]_{\text{tot}} - [\text{HSCFA}]_{\text{end}})} \cdot \frac{[\text{HSCFA}]_{\text{end}}}{([\text{SCFA}]_{\text{tot}} - [\text{HSCFA}]_{\text{end}})}. \end{aligned}$$

This again yields a quadratic equation, the solution of which is given by

$$\begin{aligned} [\text{HSCFA}]_{\text{end}} &= -1 \cdot \frac{([\text{BCE}]_{\text{tot}} + 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} \cdot ([\text{H}^+]_{\text{tot}} + [\text{SCFA}]_{\text{tot}}))}{2 \cdot (1 - 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})})} \\ &\quad \pm \sqrt{\left(\frac{([\text{BCE}]_{\text{tot}} + 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} \cdot ([\text{H}^+]_{\text{tot}} + [\text{SCFA}]_{\text{tot}}))}{2 \cdot (1 - 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})})} \right)^2} \\ &\quad + \frac{10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})} \cdot [\text{H}^+]_{\text{tot}} \cdot [\text{SCFA}]_{\text{tot}}}{(1 - 10^{(\text{pK}_{\text{SCFA}} - \text{pK}_{\text{CO}_2})})}. \end{aligned} \quad [\text{A21}]$$

After [HSCFA]_{end} has been determined from equation [A21], all other parameters follow using equations [A15] to [A19] as before.

Comparison of the Open and Closed Systems

The importance of a loss of CO₂ from the rumen for buffering of protons is intuitively clear, but can be rigorously proven using these equations. Let us compare the manner in which the system responds to a production of fermentational acid (HSCFA) in the open and the closed configuration. We will again assume initial values of

$$\begin{aligned} \text{pH}_{\text{init}} &= 6.4, \text{pCO}_2^{\text{init}} = 70 \text{ kPa}, [\text{SCFA}]_{\text{tot}}^{\text{init}} = 100 \text{ mmol} \cdot \text{L}^{-1} \\ \Rightarrow [\text{BCE}]_{\text{tot}}^{\text{init}} &= 29.49 \text{ mmol} \cdot \text{L}^{-1}, [\text{H}^+]_{\text{tot}}^{\text{init}} = 18.83 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned}$$

Assuming that an equimolar amount of SCFA and protons are generated (e.g., 20 mmol·L⁻¹), both the total concentration of SCFA and the bicarbonate excess will change according to

$$\begin{aligned} [\text{SCFA}]_{\text{tot}}^{\text{end}} &= (100 + 20) \text{ mmol} \cdot \text{L}^{-1} = 120 \text{ mmol} \cdot \text{L}^{-1} \\ [\text{BCE}]_{\text{tot}}^{\text{end}} &= (29.49 - 20) \text{ mmol} \cdot \text{L}^{-1} = 9.49 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned}$$

Using equations [A13] or [A21] as appropriate to determine [HSCFA]_{tot}^{open} and [HSCFA]_{tot}^{closed}, respectively, equations [A15] to [A18] can be used to calculate the pH, pCO₂, and total amount of free or buffered protons in the solution, yielding

$$\begin{aligned} \text{pH}_{\text{end}}^{\text{open}} &= 6.07 \quad \Delta \text{pH}_{\text{end}}^{\text{open}} = -0.33 \\ \text{pCO}_{2\text{end}}^{\text{open}} &= 70 \text{ kPa} \quad \Delta \text{pCO}_{2\text{end}}^{\text{open}} = 0 \text{ kPa} \\ [\text{H}^+]_{\text{end}}^{\text{open}} &= 22.45 \text{ mmol} \cdot \text{L}^{-1} \quad \Delta [\text{H}^+]_{\text{end}}^{\text{open}} = 3.62 \text{ mmol} \cdot \text{L}^{-1} \\ \text{pH}_{\text{end}}^{\text{closed}} &= 5.89 \quad \Delta \text{pH}_{\text{end}}^{\text{closed}} = -0.52 \\ \text{pCO}_{2\text{end}}^{\text{closed}} &= 129 \text{ kPa} \quad \Delta \text{pCO}_{2\text{end}}^{\text{closed}} = 59 \text{ kPa} \\ [\text{H}^+]_{\text{end}}^{\text{closed}} &= 39.32 \text{ mmol} \cdot \text{L}^{-1} \quad \Delta [\text{H}^+]_{\text{end}}^{\text{closed}} = 20.49 \text{ mmol} \cdot \text{L}^{-1}. \end{aligned}$$

Accordingly, the amount of [HCO₃⁻] that is required to restore the initial pH of 6.4 is much higher in the closed system than in the open system:

$$\begin{aligned} \Delta [\text{HCO}_3^-]_{\text{added}}^{\text{open}} &= 20 \text{ mmol} \cdot \text{L}^{-1} \quad \text{pCO}_{2\text{after addition}}^{\text{open}} = 70 \text{ kPa} \\ \Delta [\text{HCO}_3^-]_{\text{added}}^{\text{closed}} &= 59 \text{ mmol} \cdot \text{L}^{-1} \quad \text{pCO}_{2\text{after addition}}^{\text{closed}} = 155 \text{ kPa}. \end{aligned}$$

Calculating Base Excess

Using these equations, it is also possible to estimate the theoretical base excess BE_{6.4} and BE_{5.8} of samples as defined in the experimental part of this study by varying [H⁺]_{tot}. Assuming conditions of

$\text{pH}_{\text{init}} = 6.55$ (mean pH of hay/silage-fed animals),

$\text{pCO}_2^{\text{init}} = 70$ kPa (assumed, rumen),

$[\text{SCFA}]_{\text{tot}}^{\text{init}} = 80 \text{ mmol} \cdot \text{L}^{-1}$ (mean measured value
of hay/silage-fed animals),

$\text{pCO}_2^{\text{end}} = 100$ kPa (measurement of sample in laboratory),

$\text{pH}_{\text{init}} = 5.52$ (mean pH of concentrate/silage-fed animals),

$\text{pCO}_2^{\text{init}} = 70$ kPa (assumed, rumen),

$[\text{SCFA}]_{\text{tot}}^{\text{init}} = 89 \text{ mmol} \cdot \text{L}^{-1}$ (mean measured value
of concentrate/silage-fed animals),

$\text{pCO}_2^{\text{end}} = 100$ kPa (measurement of sample in laboratory),

$[\text{H}^+]_{\text{tot}}$ has to be increased by 0.5 and $39.5 \text{ mmol} \cdot \text{L}^{-1}$ to shift pH_{end} to 6.4 and 5.8, respectively, in the mathematical model. These values are in surprisingly good agreement with the experimental values of $0.5 \pm 3 \text{ mmol} \cdot \text{L}^{-1}$ ($\text{BE}_{(6.4)}$) and 39.2 ± 2.8 ($\text{BE}_{(5.8)}$) in this study for the hay/silage-fed group of animals. In conjunction with the good fits obtained for the buffer capacity, this suggests that the model presented here with SCFA and HCO_3^- as the major buffers adequately describes the buffering situation in the hay/silage-fed animals.

Assuming conditions reflecting the means found in the concentrate/silage-fed animals of

$[\text{H}^+]_{\text{tot}}$ has to be decreased, corresponding to an addition of a strong base. This approach yields values of -52 and $-12.6 \text{ mmol} \cdot \text{L}^{-1}$ for a pH_{end} of 6.4 and 5.8, respectively. These values deviate quantitatively from the means obtained experimentally in these samples, namely $\text{BE}_{(6.4)} = -93 \pm 18.5 \text{ mmol} \cdot \text{L}^{-1}$ and $\text{BE}_{(5.8)} = -22.2 \pm 13.8 \text{ mmol} \cdot \text{L}^{-1}$. It appears likely that other buffers than SCFA and bicarbonate are involved in buffering the pH of the rumen in this group of animals and that the total amount of bound protons found intraruminally is significantly higher than the pH might suggest.