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Method for Measuring Dissolved Hydrogen in Anaerobic Ecosystems: Application to the Rumen†

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A method of transferring dissolved H_2 to a CO_2 headspace and then absorbing out the CO_2 to concentrate the H_2 before gas chromatographic analysis was developed to measure low concentrations of dissolved H_2. A detection limit of 10 pmol of H_2 ml^{-1} of water was achieved. When used to monitor H_2 changes in a bovine rumen, a 10-fold increase in H_2 was noted 1 h after feeding and then declined rapidly to the normal steady-state concentration of 1 μM.

In methanogenic ecosystems, proton reduction leading to the formation of H_2 requires that the dissolved H_2 be maintained at low concentrations (5, 7, 9). An increase in the H_2 pool size leads to the formation of reduced fermentation end products (5, 9). In the bovine rumen, the shunting of electrons away from proton reduction to the reduction of pyruvate can lead to the sometimes fatal condition of lactic acid acidosis (1).

In methanogenic habitats, the aqueous H_2 concentration is an important parameter to monitor because of the control it exerts over the degradation of organic matter. But typical analytical methods for H_2 are relatively insensitive for measuring the low H_2 concentrations that occur in methanogenic ecosystems. Two methods designed to quantify aqueous H_2 concentrations have been described (2, 4). We report on the efficacy of a more convenient method for quantifying H_2 dissolved in aqueous phases. To illustrate use of the method, we monitored changes in the H_2 concentration of the bovine rumen in response to feeding.

Hydrogen dissolved in distilled water, artificial saliva (6), or rumen fluid was extracted by first drawing a portion (10 to 35 ml) of one of these aqueous phases into a glass 50-ml syringe (Becton, Dickinson & Co., Rutherford, N.J.) fitted with a one-way stopcock (Popper and Sons, Inc., New Hyde Park, N.Y.) and an 18-gauge needle. Carbon dioxide was then slowly drawn into the syringe until the sum of the aqueous and gaseous phase volumes equalled 50 ml. The stopcock was closed, and the syringe was vigorously shaken for 1 min. Additional CO_2 was then added to replace the CO_2 dissolved, and the syringe was shaken for another minute.

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The gas phase in the syringe was injected into the gas collection column (Fig. 1). The column had an overall length of 35 cm and an approximate volume of 25 ml. The inside diameter of the gas collection tube was 6 mm. The column was filled with a solution that contained 200 g of NaCl and 40 g of NaOH per liter. The base absorbed the CO_2, leaving the extracted H_2 (plus other gases), and the high salt concentration reduced the solubility of the gases in the filling solution.

After the gas was injected into the column, the remaining gas bubble, typically 0.5 ml in volume, was brought up to either 1.0 or 2.0 ml with air for ease of analysis. A 1-ml syringe, with attached Mininert valve (Precision Scientific, Baton Rouge, La.) and 25-gauge needle, was used to pierce the top septum and withdraw a sample of the gas bubble. With the syringe in place, the total pressure of the sample was adjusted to atmospheric pressure by adding filling solution to the side tube until the levels in the two tubes were equal. The Mininert valve was closed, and the gas sample was injected into a Carle Basic 8515 gas chromatograph (Carle Instruments, Inc., Fullerton, Calif.) equipped with a microthermistor detector, and a Porapak Q column (1.8-m by 3.2-mm inside diameter) maintained at 30°C. Argon, at a flow rate of 15 ml min^{-1}, served as the carrier gas. The H_2 retention time was ca. 60 s. When H_2 concentrations were low, the microthermistor detector signal was amplified using a 5-X operational amplifier which we had constructed. Peak areas were integrated using a Minigrator (Spectra-Physics, Santa Clara, Calif.).

The following equation was used to calculate the moles of H_2 dissolved in the sampled aqueous phase on a per-milliliter basis: $N = X[1 + \alpha(A/B)]/A$, where N = moles of H_2 ml^{-1} of extracted aqueous phase, A = volume in milliliters of the aqueous phase in the 50-ml syringe,

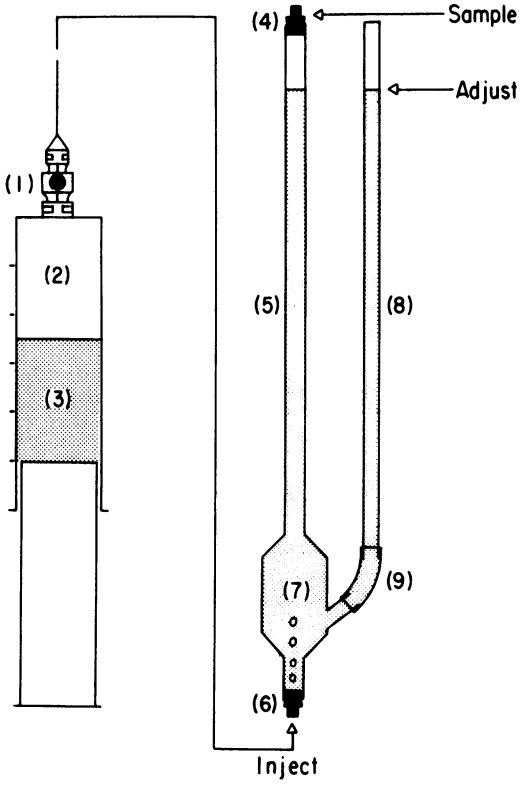

FIG. 1. *Method of H_2 extraction. (1) one-way stopcock; (2) CO_2 gas phase; (3) aqueous phase; (4) and (6) 5- by 9-mm rubber septa; (5) gas collection tube filled with NaOH and NaCl solution (7); and (8) side tube to adjust to atmospheric pressure; (9) latex tubing.*

and B = volume in milliliters of the gas phase in the syringe. The Bunsen absorption coefficient, α, for the temperature at which the extraction was carried out, was obtained from an empirical expression that describes the solubility of a gas as a function of the absolute temperature (8). In the above equation, X equals the moles of H_2 in the gas phase of the syringe at equilibrium. This value was obtained by multiplying the moles of H_2 ml^{-1} of gas in the gas bubble, as determined by gas chromatography, by the volume of the gas bubble.

To assess the accuracy of the H_2 extraction method, we drew distilled water or artificial saliva into 50-ml syringes, and added known quantities of H_2. The syringes were shaken to effect equilibration, and the H_2 was subsequently extracted by the method described above. The mean percentages of H_2 recovered for artificial saliva ranged from 85.7 to 93.7, whereas those for distilled water ranged from 98.0 to 102 (Table 1). The lower recovery of H_2 from artificial saliva

TABLE 1. *Recovery of H_2 from distilled water and artificial saliva containing known concentrations of H_2*

Aqueous phase	Calculated[a] H_2 molarity (μmol liter^{-1})	Measured H_2 molarity (μmol liter^{-1})	Mean percentage of H_2 recovery
Artificial saliva	12.9	11.7 ± 0.153 (3)[b]	90.4
	13.3	11.4 ± 0.173 (3)	85.7
	13.5	11.9 ± 0.058 (3)	88.4
	329	299 ± 2.31 (3)	90.8
	338	305 ± 3.06 (3)	90.3
	343	306 ± 3.60 (3)	89.2
Distilled water	6.95	7.00 ± 0.0235 (9)	101
	0.742	0.761 ± 0.070 (9)	102
	7.17	7.02 ± 0.233 (18)	98.0

[a] Computed assuming the aqueous phase was pure water.

[b] Mean and standard deviation; values in parentheses are the number of replications.

is probably due to the lower solubility of H_2 in this aqueous phase. However, at this ionic strength (0.22 mol·liter^{-1}), the Bunsen absorption coefficient deviates from that for pure water by ca. 6% over the temperature range (20 to 30°C) at which the extractions were performed (3). We do not know why the deviation was greater than expected, but it is not a significant concern given the sampling variability that we normally encountered.

The above procedure requires knowledge of the Bunsen absorption coefficient for H_2 in the sample being analyzed, which will often not be known because of the complexity of the sample (e.g., brackish sediments, thick sewage sludge). By rearrangement of the equation for calculation of dissolved H_2, we have found that a graphical procedure can be used which does not require knowledge of the Bunsen coefficient; the rearranged form is: $B/X = B/NA + \alpha/N$. A plot of B/X versus B/A yields a straight line with a slope of $1/N$, which is the reciprocal of the H_2 concentration, and a y-intercept equal to α/N. To demonstrate the validity of this graphical procedure, we extracted distilled water and artificial saliva containing known quantities of H_2 at six ratios of B/A. The data are plotted in Fig. 2, and the H_2 concentrations obtained for distilled water and artificial saliva were 103 and 100%, respectively, of the theoretically expected amounts, calculated using Bunsen absorption coefficients for pure water.

The H_2 pool size in the rumen of a fistulated cow fed 12 pounds (ca. 5.44 kg) of grain plus 4 pounds (ca. 1.81 kg) of hay once per day was monitored either using dialysis samplers or by obtaining samples directly. Each dialysis sam-

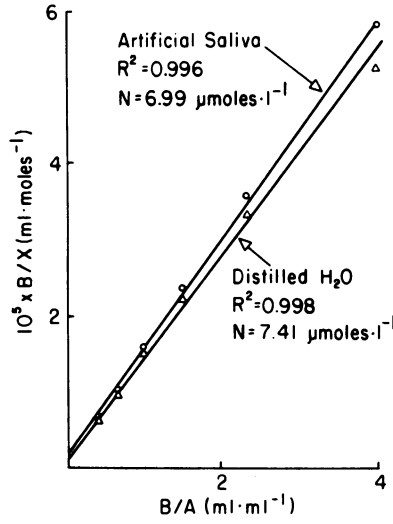

FIG. 2. *Graphical procedure for estimating H_2 concentration when Bunsen absorption coefficient is unknown. Each point is the average of three replicates.*

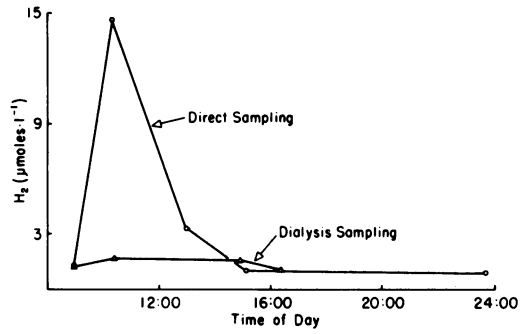

FIG. 3. *H_2 concentrations in rumen fluid determined by direct and dialysis sampling. First sample was at 9:00 a.m. just before feeding. Each point is the average of four replicates from one experiment.*

pler consisted of dialysis tubing (45- by 1.7-cm flat width) filled with 100 ml of artificial saliva and surrounded by two layers of cheesecloth. Two dialysis samplers were placed in the rumen at various times of the day. The samplers were pushed deep into the rumen contents and recovered after 60 to 90 min. We previously had determined that at least 60 min was required for the H_2 outside the bag to equilibrate with the artificial saliva inside the bag. Once the dialysis samplers were removed, 25-ml samples were taken using a 50-ml syringe, and the H_2 was extracted as described above. No difference was observed in the H_2 concentration before versus after feeding (Fig. 3).

To avoid the more lengthy equilibration period required by dialysis sampling, we obtained direct samples of rumen fluid with a 50-ml syringe to which was affixed a 1-m length of Teflon tubing (4.8-mm inside diameter). The distal end had holes (3-mm diameter) melted into it and was covered with two layers of cheesecloth which served to filter out large particles. The rumen fluid flowed freely once the flow was initiated by a slight negative pressure. The first portion was discarded before the syringe was attached to collect the sample. The syringes were immediately placed in a boiling water bath for 10 min to halt further H_2 production and consumption. After the syringes cooled to room temperature, the H_2 dissolved in the boiled rumen fluid was extracted as described above. To be certain that H_2 was not lost during the boiling step, we extracted aliquots of artificial saliva containing known amounts of H_2 after the syringes were held in a boiling bath for 10 min. This heat treatment had no effect on H_2 recovery. The H_2 pool size determined by direct sampling increased within an hour after feeding and subsequently dropped to the prefeeding concentration later in the day (Fig. 3). These results were in striking contrast to those obtained from the dialysis experiment. This initial spike of H_2 was seen repeatedly, but there was considerable variability among samples (coefficients of variation ranged from 20 to 80%). We believe the variability is due to patchiness in the ruminal H_2 concentration since the coefficient of variation of the method was generally less than 3%. The rapid increase in the ruminal H_2 concentration was previously shown to occur in sheep fed a concentrate diet (2). This dramatic rise in H_2 after feeding and the subsequent wane were missed by the dialysis sampling, apparently because equilibrium was not achieved. Even though dialysis sampling was not appropriate for this trial, it should generally be the preferred method because it removes bacteria from the sample in situ, thus avoiding the possible errors and difficulties associated with trying to inhibit bacterial activity in the collected sample. In cases where the H_2 concentration fluctuates rapidly, dialysis tubing with a higher diffusion coefficient for H_2 could be used.

The method described in this note has several advantages over the two previously described methods (2, 4). It is quicker (six samples can be extracted and analyzed in 1 h), safer, and requires less specialized glassware than the method described by Hungate (4). Its advantage over the method of Czerkawski and Breckenridge (2) is that the H_2 is concentrated by reducing the volume this gas occupies. Under the described conditions for the rumen study, the dissolved H_2 detection limit was 0.1 nmol ml^{-1}

of liquid. By increasing the aqueous sample size, withdrawing more of the bubble in the collection column, and using the most sensitive gas chromatography conditions, we achieved a detection limit of 10 pmol of H_2 ml^{-1} of liquid. This method should be applicable to analysis of gases other than H_2 since they are also concentrated by the procedure.

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