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Technique for Simultaneous Determination of [^{35}S]Sulfide and [^{14}C]Carbon Dioxide in Anaerobic Aqueous Samples†

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A technique for the simultaneous determination of [^{35}S]sulfide and [^{14}C]carbon dioxide produced in anaerobic aqueous samples dual-labeled with [^{35}S]sulfate and a ^{14}C-organic substrate is described. The method involves the passive distillation of sulfide and carbon dioxide from an acidified water sample and their subsequent separation by selective chemical absorption. The recovery of sulfide was 93% for amounts ranging from 0.35 to 50 μmol; recovery of carbon dioxide was 99% in amounts up to 20 μmol. Within these delineated ranges of total sulfide and carbon dioxide, 1 nmol of [^{35}S]sulfide and 7.5 nmol of [^{14}C]carbon dioxide were separated and quantified. Correction factors were formulated for low levels of radioisotopic cross-contamination by sulfide, carbon dioxide, and volatile organic acids. The overall standard error of the method was ±4% for sulfide and ±6% for carbon dioxide.

Recent investigations of the microbiology and chemistry of anoxic ecosystems have greatly advanced understanding of the mechanisms by which organic matter is decomposed under anaerobic conditions (1, 2, 6, 7, 9, 13–15). Because much of the organic material entering anaerobic ecosystems is ultimately dissimilated through relatively few intermediate compounds (i.e., volatile organic acids, carbon dioxide, hydrogen), determination of the rates at which these key intermediates are transformed from one chemical species to another makes possible a quantitative assessment of much of the carbon and energy flow in these habitats. In anoxic marine ecosystems, where much of the mineralization of organic matter is coupled to the anaerobic respiration of sulfate (1, 2, 5, 7, 11), measurements of the production and turnover of hydrogen sulfide and carbon dioxide are essential for quantifying terminal anaerobic metabolic processes. To this end we have developed a technique, compatible with in situ field incubations, that permits the simultaneous analysis of [^{35}S]sulfide and [^{14}C]carbon dioxide in anaerobic aqueous samples.

The glassware employed for the distillation and selective absorption of hydrogen [^{35}S]sulfide and [^{14}C]carbon dioxide from aqueous samples (Fig. 1) was constructed from two 50-ml Erlenmeyer flasks joined by 1-cm-ID glass tubing (fabricated by Anderson Glass Company, Fitzwilliam, N.H.). The basic principle of operation of the dual flask is the passive distillation of H_2S and CO_2 from an acidified dual-labeled sample (sulfur-35 and carbon-14) contained in side A over to side B, where each compound is selectively absorbed for subsequent radioisotopic analysis.

Procedures for the use of the dual flask are as follows: air is removed from the interior and connecting arm by vigorous flushing with argon gassing probes (3). A 2-ml volume of an anaerobic solution of 1 M zinc acetate is introduced into side B, and that side of the dual flask is sealed with a silicone stopper equipped with an absorption well (Fig. 1, W; Kontes Glass Co., no. K-882320-0000) containing a filter paper wick. Sample containing H_2S and CO_2 (1 to 3 ml) is introduced anaerobically via syringe into side A of the dual flask, and the latter is immediately sealed with a second silicone stopper. The sample is acidified by injecting 1 ml of anaerobic 12 N sulfuric acid through the side of the stopper in flask A via a syringe equipped with a side port needle (Hamilton, Gas Tight no. 1002). The dual flask is incubated with gentle rotary agitation for 2.0 h to permit the passive distillation of H_2S from the sample in flask A into flask B, where it is sequestered as zinc sulfide. Absorption of CO_2 is then initiated by the injection of 0.3 ml of Protosol (New England Nuclear Corp.) into well (Fig. 1, W) via a Hamilton syringe equipped with a side-port needle, and the flask is allowed to incubate with gentle agitation for an additional 2.0 h. To achieve maximum recovery of H_2S, it is essential to maintain strict anaerobiosis during the above-described procedure.

After sample incubation, the CO_2 absorption bucket is removed for radioisotopic analysis. The zinc sulfide is oxidized to the corresponding sul-

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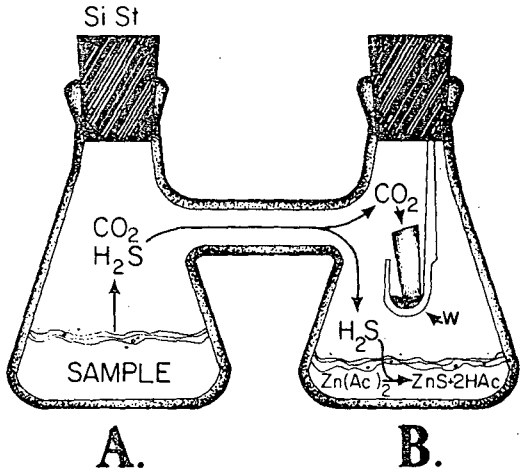

FIG. 1. *Dual flask for the passive distillation of [^{35}S]sulfide and [^{14}C]carbon dioxide from anaerobic aqueous samples. Si St, Silicone stopper; W, carbon dioxide absorption well.*

fate by treatment of the contents of side B with 0.3 ml of 30% hydrogen peroxide at 90°C for 3 min, followed by solubilization with 1 ml of 12 N sulfuric acid. Samples may then be removed for radioisotopic analysis, free of error resulting from the variable absorption of zinc sulfide to the walls of the flask and from self-absorption of the beta emissions within the granules of zinc sulfide.

Experiments investigating the dynamics of the passive distillation of hydrogen sulfide from aqueous samples into the zinc acetate absorbent demonstrated that after 60 min the recovery of sulfide was very well described by a straight line of slope 0.933 ± 0.007 and intercept 0.14 ± 0.14 (r = 0.9991). The recovery remained constant at 93% over a 2.2 order of magnitude range of added sulfide (0.35 to 50 μmol) and was not influenced by sample volume (0.5 to 3.0 ml) or by the presence of seawater. Coincidence of data obtained by iodometric titration (12) or by radioisotopic analysis permitted both approaches to be employed interchangeably for the quantification of H_2S. Provided that the total sulfide in the samples remains within the prescribed range, the radioisotopic version of this technique will permit much greater sensitivity. For example, 1 nmol of [^{35}S]sulfide, generated in anaerobic samples containing 8.9 μCi of [^{35}S]sulfate (specific activity = 0.36 μCi/μmol), was easily detectable in 0.3-ml samples of the solubilized sulfide absorption solution. A several-fold increase in sensitivity was possible by analysis of larger samples. In addition, distillation of larger aqueous samples in large-volume dual flasks (e.g., 125 ml) was possible (data not shown).

Results of studies quantifying the dynamics of the absorption of carbon dioxide into well (W) of the dual flask indicated that 2.0 h was required for the removal of carbon dioxide from the atmosphere and solutions within the dual flask. After 2.0 h of incubation, the recovery of carbon dioxide was described by a straight line of slope 0.988 ± 0.002 and intercept −0.006 ± 0.053 (r = 0.9999), provided that the levels of the gas did not exceed 20 μmol. The introduction of 50 μmol of carbon dioxide reduced the recovery from 99% to 86%. In cultures of *Desulfovibrio salexigens* containing 0.18 μCi of sodium L-[*U*-^{14}C]lactate (specific activity of the carboxyl group, 2.4 nCi/μmol), 15 nmol of produced [^{14}C]carbon dioxide could be detected. Higher sensitivity may be achieved with an increased specific activity.

For the analysis of sample containing both [^{35}S]sulfide and [^{14}C]carbon dioxide, appropriate corrections must be made for low-level cross-contamination from (i) the absorption of trace levels of [^{35}S]sulfide into the carbon dioxide absorption well and (ii) absorption of volatile ^{14}C-labeled organic compounds into both the zinc acetate solution and carbon dioxide absorption wells. Results of experiments quantifying these effects with acetate and lactate are shown in Table 1.

In samples containing [^{35}S]sulfide, [^{14}C]carbon dioxide, [^{14}C]acetate, and [^{14}C]lactate, the radioactivity observed in the zinc acetate solution (D_{ZA}) may be expressed:

$$D_{ZA} = a(D_S) + d(D_A) + g(D_l) \quad (1)$$

where D_S = [^{35}S]sulfide present in the sample; D_A = [^{14}C]acetate present in the sample; D_l = [^{14}C]lactate present in the sample; a = efficiency of the passive distillation and absorption of sulfide; and d and g = moles of [^{14}C]acetate and [^{14}C]lactate dissolved in the zinc acetate solution per mole present in the sample, respectively.

Similarly, the radioactivity observed in the carbon dioxide absorption well (D_W) is described by the equation:

$$D_W = e(D_C) + c(D_A) + b(D_S) + h(D_l) \quad (2)$$

where D_C = [^{14}C]carbon dioxide present in the sample; b = moles of [^{35}S]sulfide absorbed in the carbon dioxide well per mole in the sample; c and h = moles of [^{14}C]acetate and [^{14}C]lactate sequestered in the carbon dioxide absorption well per mole in the sample, respectively; and e = efficiency of distillation and absorption of [^{14}C]carbon dioxide.

Data for each of these parameters may be found in Table 1. The enhanced volatility of lactate relative to acetate was the result of trace volatile substituents present in the isotope prep-

TABLE 1. *Factors relating the efficiency of passive distillation and the degree of cross-contamination of label within the dual flask*[a]

Labeled compound	Amt present in dual flask (μmol)	Factors[b]							
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
[^{35}S]sulfide	0.35–50	0.933 (±0.007)	3.7 (±0.5) × 10^{-3}	—	—	—	—	—	—
[^{14}C]acetate	0.004–1.0	—	—	6.18 (±0.24) × 10^{-4}	3.17 (±0.31) × 10^{-3}	—	—	—	—
[^{14}C]acetate	1.0–31	—	—	1.29 (±0.06) × 10^{-3}	3.12 (±0.05) × 10^{-3}	—	—	—	—
[^{14}C]lactate	0–12.5	—	—	—	—	—	—	1.92 (±0.02) × 10^{-2}	7.10 (±0.01) × 10^{-2}
[^{14}C]lactate	12.5–25	—	—	—	—	—	—	2.19 (±0.01) × 10^{-2}	5.66 (±0.16) × 10^{-1}
[^{14}C]carbon dioxide	1.0–20	—	—	—	—	0.988 (±0.002)	0	—	—

[a] Sample solutions (2.5 ml) containing zinc [^{35}S]sulfide, sodium [*U*-^{14}C]acetate, sodium [*U*-^{14}C]lactate, or sodium [^{14}C]bicarbonate in the ranges indicated were incubated in dual flasks as outlined in the text. Moles of labeled compound trapped in the CO_2 well or zinc acetate solution were computed from the known specific activities and plotted against the amounts present in the original sample. Parameters *a* to *h* were obtained as the slope of the best-fit straight line through the data. In all cases the intercept was negligibly different from zero and the linear correlation coefficients were between 0.95 and 0.999. The values in parentheses represent the standard deviation.

[b] Factors (expressed per mole of respective substance present in the sample): *a*, moles of sulfide recovered in the zinc acetate solution; *b*, moles of sulfide absorbed in the CO_2 absorption well; *c*, moles of acetate sequestered in the CO_2 absorption well; *d*, moles of acetate dissolved in the zinc acetate solution; *e*, moles of CO_2 recovered in the absorption well; *f*, moles of CO_2 retained in the zinc acetate solution; *g*, moles of lactate dissolved in the zinc acetate solution; *h*, moles of lactate sequestered in the absorption well. —, Not applicable.

aration. The degree of label carry-over can vary several-fold with the lot of [^{14}C]lactate employed, necessitating independent calibration. The volatile substance appeared to be nonacidic in character in that the level of cross-contamination was independent of the pH of the sample contained within the dual flask (pH <1, 3.0, 8.0).

The true levels of [^{35}S]sulfide in the zinc acetate solution and [^{14}C]carbon dioxide in the absorption well may be determined by solving equation (1) for D_S and substituting into equation (2):

$$D_S = \frac{1}{a}[(D_{ZA})] - d(D_A) - g(D_l)] \quad (3)$$

$$D_C = \frac{1}{e}\left[(D_W) - \frac{b}{a}(D_{ZA}) - \left(c - \frac{bd}{a}\right)D_A - \left(h - \frac{bg}{a}\right)D_l\right] \quad (4)$$

From Table 1, bd/a and bg/a are negligible relative to c and h, respectively, and equation 4 may be simplified to:

$$D_C = \frac{1}{e}\left[(D_W) - \frac{b}{a}(D_{ZA}) - c(D_A) - h(D_l)\right] \quad (5)$$

An analysis was performed to determine the error propagated from the uncertainties of the independent variables in equations (3) and (5). The standard error of D_S was ±4% under most circumstances and resulted from the error in measuring D_{ZA} (average ± 3.6%) and, to a lesser extent, the error in determining the efficiency of distillation of sulfide (a) and in measuring D_{ZA}.The standard error typical for D_C was ±6% and resulted predominantly from the errors associated with the measurement of D_W (average 3.4%), and the error in determining the extent to which [^{35}S]sulfide is absorbed into the carbon dioxide absorption well (b).

For demonstrating the utility of the dual flask we measured the production of hydrogen [^{35}S]-sulfide and [^{14}C]carbon dioxide from a [^{35}S]sulfate- and [^{14}C]lactate-labeled culture of *D. salexigens*, and we compared the results with data obtained from iodometric (H_2S) and gas chromatographic (CO_2) analyses of the culture. The isotopic data were corrected for cross-contamination using equations (3) and (5) and the data in Table 1. The values obtained by these different methods compared well in six of eight cases (Table 2). The instances where significant differences were observed (hydrogen sulfide, 13.3 h; carbon dioxide, 16.4 h) are nonsystematic.

TABLE 2. *Comparison, using the dual-flask technique and conventional analytical techniques, of hydrogen sulfide and carbon dioxide production by D. salexigens*[a]

Time (h)	Sulfide produced (mM)		Carbon dioxide produced (mM)	
	^{35}S dual flask	Iodometric titration	^{14}C dual flask	Gas chromatography
10.1	0.49 (±0.03)	0.53 (±0.05)	0.82 (±0.05)	0.66 (±0.07)
13.3	0.69 (±0.05)	0.86 (±0.08)	1.39 (±0.08)	1.43 (±0.17)
16.4	1.24 (±0.08)	1.10 (±0.10)	2.13 (±0.14)	1.68 (±0.20)
19.3	1.30 (±0.09)	1.30 (±0.12)	3.00 (±0.18)	3.11 (±0.37)

[a] Analyses were performed on a culture of *D. salexigens* growing in an anaerobic artificial seawater medium initially containing 9.5 mM lactate, 9.5 mM sulfate, and 1.63 mM carbon dioxide. Sodium L-[*U*-^{14}C]lactate and $H_2{}^{35}SO_4$ were present in the medium at final specific activities of 7.66 μCi/mmol and 174 μCi/mmol, respectively. The cell density at zero time was 2.2×10^6 cells per ml. Samples (2.5 ml) were analyzed for $H_2{}^{35}S$ and $^{14}CO_2$ using the dual flask, and the data were corrected for cross-contamination. Sulfide recovered from 2.5-ml samples in the dual flask was also measured via iodometric titration (12), and carbon dioxide in 2.5-ml acidified samples was analyzed using headspace gas chromatographic procedures (separation of CO_2 was effected on a Porapak QS column [0.125 in. by 6 ft, ca. 6.4 mm by 1.83 m] at 85°C using helium at 15 ml/min as the carrier gas; detection was by thermal conductivity). Sulfide and carbon dioxide produced were determined from the differences in concentration of these compounds at indicated times and those present at zero time. The values in parentheses are the standard errors propagated from the uncertainties in the independent variables used in this computation. Correction for the fractionation of sulfur-35 by sulfate-reducing bacteria (10) was made by multiplying the radioisotopic data by 1.06.

Data from analogous experiments (not shown in Table 2) reflected a favorable comparison between the radioisotopic and chemical techniques at these levels of hydrogen sulfide and carbon dioxide. At the levels of ^{35}S and ^{14}C present in the *Desulfovibrio* experiments, the major source of cross-contamination within the dual flask resulted from the absorption of [^{35}S]sulfide into the carbon dioxide absorption well, comprising 15% of the total radioactivity observed in the well. The contribution of [^{14}C]acetate and [^{14}C]lactate to the observed radioactivity in the zinc acetate solution and the carbon dioxide well was not large in these experiments.

In addition to permitting the simultaneous analysis of hydrogen sulfide and carbon dioxide, the dual-flask technique results in a high recovery of sulfide characteristic of active distillation procedures (4, 5), while maintaining the ease and convenience of passive distillation methods (8). The dual flask has been utilized in detailed laboratory studies of the carbon and sulfur metabolism of sulfate-reducing bacteria as well as in field studies of anaerobic habitats where simultaneous measurements of the concentration and rates of turnover of H_2S and CO_2 are highly desirable.

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