Technique for Measuring $^{14}$CO$_2$ Uptake by Soil Microorganisms In Situ

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Uptake of $^{14}$CO$_2$ in soils due to algae or sulfur-oxidizing bacteria was examined by incubation of soil samples with gaseous $^{14}$CO$_2$ and subsequent chemical oxidation of biologically fixed radioactive isotope to $^{14}$CO$_2$ for detection with a liquid scintillation counting system. The $^{14}$CO$_2$ was added to the soil in the gas phase so that no alteration of the moisture or ionic strength of the soil occurred. Wet oxidation of radioactive organic matter was carried out in sealed ampoules, and the $^{14}$CO$_2$ produced was transferred to a phenethylamine-liquid scintillation counting system with a simply constructed apparatus. The technique is inexpensive and efficient and does not require elaborate traps since several possible interfering factors were found to have no harmful effects. Experiments in coal mine regions and in geothermal habitats have demonstrated the ecological applicability of this technique for measurement of CO$_2$ fixation by sulfur-oxidizing bacteria and soil algae.

Observation and quantification of microorganisms in nature are difficult tasks, particularly in soil habitats, due to the physical structure of soil particles and the small number of organisms associated with these particles. Some of these problems have been overcome by the use of fluorescent antibodies (10) or scanning electron microscopy (8), or by specialized techniques with the light microscope (6). Fluorescent antibodies have been useful for direct observation of specific organisms, whereas scanning electron microscopy and the inverted microscope technique have allowed observation of soil organisms in situ. However, none of these methods permits a direct study of the metabolic activity of the organisms observed.

One of the best ways of measuring microbial activity in situ is by the use of radioactive isotopes (5), but, to date, radioactive isotopes have been used almost exclusively in aquatic ecosystems. The reason for this is that processing of samples for counting of incorporated radioactivity is much easier in water than in soil habitats. The technique described here provides an inexpensive and relatively simple wet oxidation method of assessing the incorporated radioactivity in soil. Since the isotope is added as gaseous $^{14}$CO$_2$, no alteration in the ionic strength or moisture content of the soil occurs. Hubbard et al. (9) described a similar procedure for measuring $^{14}$CO$_2$ fixation in soil, designed to be used in the exploration of Mars, but their technique is considerably more complicated and expensive than the one described here.

MATERIALS AND METHODS

Incubations. Soil samples were placed in tared 5-ml serum bottles (Wheaton Glass Co.), capped with rubber serum stoppers and preincubated for 20 min at the desired temperature of incubation. The use of tared serum bottles allowed subsequent determination of the exact mass of soil which was incubated. Samples for chemolithotrophic detection (1 to 2 g per bottle) were maintained in the dark during the incubation period by placing the sample bottles in 35-mm film cans. Samples for photosynthetic studies (0.2 to 0.5 g per bottle) were incubated in the light, whereas the dark controls were serum bottles wrapped in two layers of aluminum foil. Field incubations took place in a thermal effluent issuing from a hot spring at Roaring Mountain in Yellowstone National Park which provides a gradient of temperatures from 25 to 90 C. Laboratory incubations took place in constant temperature water baths with illumination provided by fluorescent lighting at about 900 foot candles. After the preincubation period, 0.5 ml of air was removed from each bottle with an air-tight syringe. Each incubation was then begun by the injection of 0.5 ml of gaseous $^{14}$CO$_2$ into the bottle through the serum cap. Problems which could arise from an internal pressure greater than that of the atmosphere such as leakage of $^{14}$CO$_2$ or incuba-
tion of organisms under abnormal pressure were thus avoided. Incubations were stopped by the addition of 2 ml of 1 N perchloric acid (PCA). In the field, the PCA addition was made after the removal of the serum cap, thus flushing a large amount of the unincorporated $^{13}CO_2$ from the bottle while still outdoors. In the laboratory, PCA was injected through the serum cap. As a safety precaution, the unincorporated $^{13}CO_2$ was flushed from serum bottles only in an operative fume hood. All samples were processed in the laboratory.

$^{13}CO_2$ Generation. The $^{13}CO_2$ was generated from NaH$^{13}CO_3$ in a simple device consisting of a CO$_2$-impermeable rubber membrane (Trojans, Youngs Drug Products Corp., Piscataway, N.J.) placed within a 60-ml polyethylene bottle which had two 3-mm holes cut in its sides. The top of the membrane was folded over the outside of the mouth of the bottle and sealed with an airtight rubber serum cap.

The membrane was filled with air, and a needle was inserted through the cap into the membrane to allow the internal and external pressures to equilibrate. After equilibration, 3 ml of air was removed, and 2 ml of 10 N H$_2$SO$_4$ was injected through the cap into the membrane. Then 1.0 ml of NaH$^{13}CO_3$ (New England Nuclear Corp., Boston, Mass.) was injected into the membrane and was converted to $^{13}CO_2$ by reaction with the H$_2$SO$_4$. The specific activity of the NaH$^{13}CO_3$ injected was 1 $\mu$Ci/10 $\mu$Ci and either 10 $\mu$Ci or 20 $\mu$Ci was injected. The $^{13}CO_2$ thus generated should be withdrawn immediately for sample injection to minimize the possibility of $^{13}CO_2$ leakage through the membrane.

The $^{13}CO_2$ was transferred from the generating bottle to the sample bottles with a 1-ml airtight syringe (Becton, Dickinson & Co., Rutherford, N.J.). The exact amount of $^{13}CO_2$ injected into the samples was measured in a liquid scintillation counter after the injection of a fraction of $^{13}CO_2$ into a scintillation vial containing the appropriate scintillation fluid (see below).

The polyethylene bottle protected the fragile membrane from physical damage, and the holes in the bottle kept the membrane exposed to atmospheric pressure at all times. As samples were withdrawn, the atmospheric pressure caused the volume of the membrane to decrease by the amount of the sample withdrawn. This constant equalization of internal and external pressures meant that all samples withdrawn had the same activity of $^{13}CO_2$ per unit volume.

Processing and oxidation. After incubation, samples were transferred from the serum bottles to 20-ml (Kimble Products) or 50-ml funnel-top glass ampoules (Wheaton Glass Co.). Radioactive carbonate formed nonbiologically in the soil was removed by treating the sample with a carbonate flushing solution prepared by mixing 57 ml of concentrated H$_2$SO$_4$ with 92 g of FeSO$_4$·7H$_2$O in 600 ml of distilled water (3). The soil particles were transferred into the ampoules with the carbonate flush solution with a squeeze bottle. It was imperative that the total volume of carbonate flush solution for this transfer be kept to a minimum, preferably 2 to 3 ml, since volumes greater than 3 ml produced severe problems during the boiling and sealing steps described below. Samples were rapidly brought to the boiling point in a 300°C sand bath and kept at a slow boil for exactly 90 sec to drive off unincorporated $^{13}CO_2$ from the liquid phase (3). The ampoules were then flushed with air for about 10 sec to insure the total removal of $^{13}CO_2$ from the air phase of the ampoule. The fact that the carbonate flush solution is 2 N insures that $^{13}CO_2$ is the favored species, whereas the FeSO$_4$ provides ferrous ions at a concentration of 2.8% (w/v), thus protecting the organic matter from oxidation during the boiling (4).

Dissolved acid was then added to the ampoules, 5 ml to the 20-ml ampoules and 10 ml to the 50-ml ampoules. This acid was prepared by mixing concentrated H$_2$SO$_4$ and 85% H$_3$PO$_4$ in a 6:4 ratio, cooling, and storing in a glass-stoppered reagent bottle (3). Approximately 1 g of reagent grade K$_2$Cr$_2$O$_7$ was added to each ampoule and washed into the ampoule with a small amount of water. The ampoules were immediately sealed with either an oxygen-methane torch or a propane torch, cooled, checked for leakage by inversion, and autoclaved at 121°C, 15 psi for 60 min to oxidize the organic matter in the samples to CO$_2$. After being autoclaved, the samples were processed as described below.

The processing apparatus was a modification of that described by Strickland and Parsons (11) and consisted of a large rubber tubing sleeve, the inside diameter of which was slightly smaller than the outside diameter of the ampoule (Fig. 1). The sleeve was closed at the top with a rubber stopper through which a glass tube and a stainless steel cannula [0.063-inch (1.6-mm) internal diameter] extended into the sleeve. The cannula was connected to an air pump (Marco, J. B. Maris Co., Bloomfield, N.J.) which constantly forced air into the sleeve. $^{13}CO_2$ was flushed from the ampoule through the sleeve via the glass tube and was transmitted to two scintillation vials connected in series. The output tube from the second vial led directly into the air with no further connection. The processing itself was begun by inserting an ampoule into the rubber sleeve and securing it with a hose clamp, thus creating an airtight sleeve. Scintillation vials were screwed into the caps and the air flow was adjusted so that there were 5 to 10 bubbles per sec in the scintillation vials. The hose connecting the sleeve to the vials was then briefly clamped and the top of the ampoule was broken off by pressure applied from outside the sleeve. The clamp was quickly but gradually released, minimizing pressure buildup in the sleeve, and the cannula was inserted through the hole in the top of the ampoule into the liquid phase of the ampoule. The ampoule was flushed for 5 min, insuring complete $^{13}CO_2$ removal. The first scintillation vial was removed, capped, and counted. Another vial was put in this position, and the system was "washed" by passing of air for another 2 min to eliminate cross-
contamination between samples. After this 2-min bubbling, the ampoule was removed, and the cannula was rinsed in water for about 10 sec. The "wash" vial was replaced, the next ampoule to be processed was inserted, and the procedure was repeated. Only the first vial per sample was counted since virtually all the radioactivity was trapped in the first vial. The second vial was used to reduce even the trace amounts of $^{14}$CO$_2$ which would otherwise be emitted into the laboratory. This second vial was changed after every 100 processings, whereas the wash vial was changed after every 50 samples.

Scintillation counting. The composition of the scintillation fluid used for the absorption of $^{14}$CO$_2$ was a modification of that used by Woeller (12). Each scintillation vial contained 10 ml of toluene-fluor mixture with 0.375 g of 2,5-diphenyloxazole (PPO, Beckman Instruments, Inc., Fullerton, Calif.) and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (POPOP, Packard Instrument Co., Inc.) per 1,000 ml of toluene. In addition, each vial contained 2.5 ml of a mixture of one part of Nuclear-Chicago solubilizer (NCS) to absorb water and two parts of phenethylamine (Fisher Scientific Co.) to trap the $^{14}$CO$_2$ plus two parts of analytical grade methanol (0.06% water, Fisher Scientific Co.) to aid in solubilizing the phenethylamine.

Samples were counted with Beckman LS-100, LS-233, or B-Mate II liquid scintillation systems and corrected for background radiation.

Cultures. Sulfur-oxidizing bacteria were isolated from coal mine regions and thermal regions. Sulfolobus, a new acidophilic thermophilic sulfur-oxidizing bacterium will be described elsewhere. It was grown at 55°C in Allen’s salts medium (1) at pH 3.0 with 1% sterile sulfur. Thermophilic Thiobacillus cultures were isolated in the same manner.

The Cyanidium caldarium culture was obtained from W. N. Doemel and maintained in Allen's salts medium at pH 3.0 in the light, the culture being gassed with 5% (v/v) CO$_2$ in air.

RESULTS

Development of the technique. The efficiency of the technique in oxidizing various compounds was examined, and the results are given in Table 1. Varying concentrations of uniformly labeled [1$^4$C]glucose and [2-$^1$C]-uracil were added to ampoules, oxidized, and processed as described above. The activity of the added glucose and uracil was measured by pipetting samples into scintillation vials containing scintillation fluid as previously described. C. caldarium cells were labeled by growth in $^{14}$CO$_2$ for 1 hr at 45°C, and the washed samples were added to ampoules. In all cases, the procedure was performed on samples containing 0.5 g of soil (from Roaring Mountain in Yellowstone National Park) per ampoule to reproduce the treatment of natural samples as closely as possible. An excess of either nonlabeled glucose or uracil was added to the appropriate ampoules. The activity of C. caldarium was determined by filtering a sample onto a 0.45-μm membrane filter (Millipore Filter Corp.), air-drying the filter, placing it in scintillation fluid with standard primary and secondary flours (PPO and POPOP, respectively), and counting it with a liquid scintillation system. As seen in Table 1, 61 to 87% of the added radioactivity was recovered during the oxidation of glucose and uracil, whereas 98% of the added radioactivity was recovered during the oxidation of C. caldarium. The recovery efficiency was independent of the amount of radioactivity present. The incomplete recovery of $^{14}$CO$_2$ from oxidized $^{14}$C organic compounds should be noted. These results do not detract from the usefulness or efficiency of the technique. This technique was not designed to determine the total organic matter present in a given soil but rather the amount of biologically fixed $^{14}$CO$_2$. The more important result, therefore, is the virtually complete recovery of $^{14}$CO$_2$ from $^{14}$C-labeled C. caldarium cells.

The technique was examined for the possible interfering effects of nonradioactive carbon
dioxide, chloride, and water. It is necessary to show that the phenethylamine in the concentration used is not saturated by nonradioactive CO₂ from the air during the 5-min aeration process. Scintillation vials containing the complete scintillation fluid were aerated with 5% CO₂ in air for varying time periods. Ten ampoules containing equal amounts of [¹⁴C]-glucose were then oxidized and processed using the aerated scintillation vials. During processing, air was scrubbed free of CO₂ by passing it through an ascarite (A. H. Thomas Co.) tower. Even 5 min of aeration with CO₂-enriched air had no inhibitory effect on the subsequent ability of phenethylamine to absorb radioactive CO₂ released from [¹⁴C]glucose.

Because many CO₂-absorbing agents also trap Cl₂ (2), the effect of NaCl in the sample on CO₂ absorption by phenethylamine was examined. Chlorine is created during the oxidation process if Cl⁻ is present in the sample (3). Constant amounts of [¹⁴C]glucose (0.1 ml of 0.1 μCi/ml, 1.67 μCi/μg) were oxidized with varying amounts of NaCl. The recovery of ¹⁴CO₂ was not reduced unless the concentration of NaCl exceeded 20% (w/w). Such levels of NaCl would be found only in extremely saline soils.

Finally, the effect of water on the scintillation counting efficiency was measured by means of the [¹⁴C]toluene internal standard method. The complete scintillation fluid was prepared using a constant amount of [¹⁴C]toluene (0.1 ml of 0.5 μCi/ml) in each vial. Various amounts of water were added to each vial, and the effect of counting efficiency was measured. Vials with more than 0.15 ml of water became turbid, but the counting efficiency was not affected even at water concentrations much higher than this. Evidence that even small amounts of water are not transmitted to the scintillation vials is that a trap containing indicator MgCO₃ (Drierite, W. A. Hammond Co.) was placed between the rubber sleeve and the first scintillation vial during the processing of about 100 samples, and no color change was detected.

### Applicability of the technique
The following two experiments demonstrate the applicability of this technique for studying microbial autotrophic activity in soils. Samples for chemoautotrophic studies were obtained from a sulfur-rich soil on Roaring Mountain in Yellowstone National Park where both Thiobacillus and Sulfolobus have been isolated routinely, whereas samples for photoautotrophic studies came from Amphitheater Springs in Yellowstone National Park.

A soil from Roaring Mountain sterilized in dry heat for 3 hr at 300 C was placed in 5-ml serum bottles. The sterile soil was inoculated with 8 × 10³ bacteria from a culture of Sulfolobus. The samples were preincubated at 55 C, and the radioactive CO₂ (163,000 counts per min per sample) was injected. Three sets of serum bottles were used in the experiment. Those containing autotrophs plus sterile soil were incubated for 8 hr, and PCA was added at various time intervals. After PCA was added, the sample was held at the temperature of incubation for the full incubation period. A second set of samples containing autotrophs plus sterile soil was incubated for 1 hr, and then the incorporation of ¹⁴CO₂ was stopped with PCA. The third set containing only sterile soil served as the control. Figure 2 demonstrates that CO₂ uptake by sulfur-oxidizing autotrophs inoculated into sterile soil was detected by this technique. The figure also shows that ¹⁴CO₂ incorporation is stopped by the addition of 1 n PCA and that heat-sterilized soils show no autotrophic ¹⁴CO₂ fixation.

A sample of soil from Amphitheater Springs
DISCUSSION

The technique described is valuable for in situ ecological studies in soils. The wet oxidation method reproducibly oxidizes whole organisms as well as selected compounds of biological importance. The processing method is simple and efficient. The technique is sensitive since all of the $^{14}$CO$_2$ which is incorporated into organic material is released as $^{14}$CO$_2$ upon oxidation. Thus even samples with low levels of incorporation can be accurately determined.

Soil microorganisms are very sensitive to changes in the moisture content and ionic strength of their environment. The incubation of samples with gaseous $^{14}$CO$_2$ allows the observation of microbial activity under in situ conditions since neither the moisture content nor the ionic strength of the sample is altered.

Although there are many methods presently available for studying autotrophic CO$_2$ fixation in aquatic systems (11), there have been no convenient methods for assaying this activity by soil autotrophic organisms. This technique allows a simple extension of primary productivity studies to include the contribution made by these soil autotrophs.

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![Fig. 2. $^{14}$CO$_2$ uptake in sterile soil amended with sulfur-oxidizing bacteria. Uptake of $^{14}$CO$_2$ was stopped by the addition of 2 ml of 1 N PCA. (●) No PCA added; (○) PCA added after 1 hr; (x) sterile control.](image1)

which contained the alga C. caldarium was collected and distributed into four sets of six serum bottles each. In each set, triplicate samples were incubated in the light and duplicate samples were incubated in the dark, along with a light control which had 2 ml of 1 N PCA added at the beginning of the incubation period. This last serum bottle was used to demonstrate that all $^{14}$CO$_2$ fixation which occurs in these samples is a biological process. All serum bottles were incubated at 45 C, the optimum for this alga, (7) with 340,000 counts/min of $^{14}$CO$_2$ as previously described. The incorporation of $^{14}$CO$_2$ was stopped by the addition of PCA at 1, 2, 3, and 4 hr. Figure 3 shows that the incorporation of $^{14}$CO$_2$ increases linearly as a function of time for 2 hr, and incorporation continues at a reduced rate beyond this point.

This technique has also been employed in coal mine regions to evaluate the activity of iron-oxidizing autotrophs in coal refuse piles. The applicability is not limited to acidic environments however. Samples of greenhouse soil (pH 8) containing algae demonstrated a substantial light-stimulated $^{14}$CO$_2$ uptake by this method. Uptake of CO$_2$ by heterotrophic microorganisms probably occurs in many soil systems. In our work, heterotrophic uptake has not been a source of confusion, either because the autotrophs have been present in very large numbers or, in the case of the photoautotrophs, because dark control values can be deducted from those of samples incubated in the light.

![Fig. 3. Photoautotrophic incorporation of $^{14}$CO$_2$ as a function of time. Incubations were at 45 C and were stopped by the addition of 1 N PCA. To each bottle was added $^{14}$CO$_2$ (340,000 counts per min). (●) Light incorporation (average of three determinations); (○) dark incorporation (average of two determinations); (x) PCA control (one determination).](image2)
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