Method for Simultaneous Measurement of Radioactive and Inactive CO₂ Evolved from Soil Samples During Incubation with Labeled Substrates

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An apparatus is described which allows the simultaneous, continuous, and highly sensitive analysis of inactive and radioactive CO₂ evolved from 14C-supplemented soils or other materials. The apparatus consists of a control unit, a commercially available conductometric CO₂ analyzer, and a fraction collector. A number of model experiments were conducted to demonstrate the potentials of the apparatus. These included analysis of the time course of priming action, when 14C-glucose was added to soil, separation of CO₂ respiration peaks caused by simultaneous degradation of radioactive and inactive soil supplements, and study of the effects of a fungicide, Benomyl, on degradation of 14C-labeled glucose. In the last experiment, partial degradation of the fungicide could also be followed.

The production of CO₂ or the uptake of O₂ is frequently used to measure the extent and duration of oxidation of organic substrates in soil. The measurements obtained always represent the sum of single processes which cannot, with certainty, be separated by using conventional respirometric methods. By using 14C-labeled compounds and by simultaneously measuring quantities of inactive and radioactive CO₂ produced, it becomes possible to separate the total CO₂ produced into its constituent parts.

The procedure described below allows the simultaneous, continuous, and highly sensitive measurement of both inactive and radioactive CO₂. Existing methods for radiorespirometry in modified Warburg flasks followed by scintillation counting (6), proportional flow-type counters (5), end-window counters (2, 3), gas ionization chambers (4, 6), or Ba¹⁴CO₂ planchets (1, 3) have disadvantages which, in part, include small sample size, lower sensitivity, and a lesser degree of automation.

MATERIALS AND METHODS
Apparatus and functions. The apparatus used in this work (Fig. 1) consists of a commercially available analyzer for inactive CO₂, a recorder, a switching device with inlets for six samples (Wösthoff Co., D 463 Bochum, FR Germany), an absorption cell for radioactive CO₂, a fraction collector, and a control unit.

Moist, CO₂-free air was pumped through a soil sample, through the switching device (2), and then into the gas analyzer (3). In the analyzer, the conductivity of a 0.05 N NaOH solution was measured before and after its reaction with CO₂. The gas produced in each soil was measured for a total of 10 min once every hour. Results were recorded as percentage of CO₂. The gas which accumulated during the remaining 50 min was dried (M1) and pumped into an absorption cell, where the CO₂ was collected in 30 ml of methanol-ethanolamine (7:3). A fritted glass outlet insured the even distribution of the gas in the trapping solution. Ten milliliters of this solution was then mixed with 10 ml of scintillation solution [4 g of 2, 5-diphenyloxazole plus 0.3 g of 1, 4-bis-2'-(4-methyl-5'-phenyloxazolyl)-benzene in 1 liter of toluene solution] and counted in a Nuclear Chicago Mark II spectrometer for 10 min.

The flow of trapping solution from a reservoir (Rv2) into the CO₂ absorption cell was governed by a solenoid-operated pinch clamp (PC4). A photoelectric level detector (LD1) directed through the CO₂ absorption cell regulated the quantity of methanol-ethanolamine entering the cell. Draining of the absorption cell into the fraction collector was controlled by a solenoid-operated pinch clamp (PC3).

After a 10-min flow of ¹⁴CO₂ through the apparatus,
At the gas lines and the CO₂ absorption cell contained radioactive residues. To remove ¹⁴CO₂ gas residues, non-radioactive gas from the following (unlabeled) soil sample was used to flush the system. After 5 min of flushing, the gas flow into the cell was interrupted, and for the remaining 5 min, the gas was directed into a trap (F12). After removal of the reacted trapping solution, the cell was flushed with fresh solution and then immediately refilled for collection of ¹⁴CO₂ from the next soil sample. The duration of one revolution of the cyclic program was 20 min (Fig. 2).

Control unit. The control unit, in connection with the switching device, regulated the operations of the cyclic program. Both were equipped with synchronized electric motors, which drove barrel controllers with program disks (Fig. 2). The barrel controllers could be replaced by digital logic and inexpensive integrated circuit timers. Synchronization was over the electrical mains. The motor in the control unit received its current through cable 5, directly from the switching device. To initially synchroize the system, the program in the control unit was manually interrupted (Fig. 3, button b2) until the program in the switching device reached its starting point, i.e., when the device switched to the first soil sample containing a ¹⁴C-labeled substrate.

The impulse that initiated the program originated in the switching device. It was led to the control unit (wire 3 of cable 5) where it caused the first switching of the bistable relay r1 (PC1 opened). Dissynchrony of r1 was corrected by pushing b1. The closing of PC1 was controlled by program disk PD1 (Fig. 2). During the normal operation of the Wösthoff apparatus, whereby only unlabeled CO₂ was measured, a switching impulse was given every 10 min. For the purpose of simultaneous measurement of ¹⁴CO₂ and CO₂, alternating impulses from the switching device were overidden by program disk PD1.
The second switching of relay r1, which was initiated by program disk PD2, caused PC1 to close and PC2 to open. Simultaneously, the photoelectric level detectors and the first switching circuit (released by program disk PD3) received current (PC3 opened). As program disk PD3 activated the second switching circuit (PC3 closed; PC4 opened), photoelectric level detector (LD1) also became operative. The rise of the trapping solution in the absorption cell interrupted the photoelectric level detector and closed PC4. To avoid repeated activation of the relays in s1 during absorption of CO₂ (due to bubble formation), the relay r3 went into a holding position which was overridden when program disk PD3 returned to the first switching circuit. The procedure for rinsing the cell was the same. The self holding of relay r3 was terminated when r1 switched. The program disk PD4 gave the switching impulse to the fraction collector over cable 6.

Safety precautions. As a safety precaution, malfunction in any part of the system causes the entire apparatus to automatically shut down. Since a temporary interruption of the AC supply could cause a disruption of the switching order, the feed-in is led over the self-contained circuit remote control switch (m). To re-start the machine, button b4 must be pushed; b3 is used to shut the machine off at the end of normal operation. To prevent possible leakage of “CO₂ into the atmosphere during unintentional shut-down of the machine, all pinch clamps close when shut off.

Together with power supply p2, switching unit s3 serves to charge the photoelectric level detectors (connections 7 and 9). The lamp current is simultaneously controlled by the transistor-amplifier switch. Malfunction of one or both lamps leads to interruption of the self-holding of the circuit remote control switch through relay r4. In case LD1 fails to work and PC4 does not close, CO₂ trapping solution overflows into the safety cell SC. The photoelectric level detector LB2 in the safety cell responds (in connection with the switching device s2, the thermometer R2, and the relay r2), and the entire apparatus shuts down.

The circuit of the pinch-clamp PC2 is simultaneously the feed-in for p1, s1, s2, s3, and the program disk PD3. The switching position of the relays in s1 and s2 is in a resting stage, when the light barrier is interrupted. It starts operating a finite time after turning on the feed-in. This is due to time constants which originate from the filaments and the photore sistors. As far as the operation of s1 is concerned, this is without importance. The switching position of the relay becomes of importance only after PD3 moves into the second switching circuit (activation of PC4).

Bridging of the circuits of s2 is over thermometer R2 (resistor with a large negative temperature coefficient). The high cold resistance of the thermometer hindered the immediate activation of relay r2. The relay is first turned on when the resistance diminishes due to warming up after interruption of the light barrier.

![Wiring diagram of the control unit](https://example.com/wiring-diagram.png)
Substrates. Since the described method was part of a contribution to the International Biological Program, the soil used was collected from the humus layer of a beech forest (German IBP site “Solling”). The C content of the soil was 21.68%, and the pH (KCl) was 2.90. The method is equally applicable to soils of other pH ranges and lower C contents. At the beginning of each experiment, 30 g of soil (oven dry weight; particle size ≤2 mm) were moistened with 20 ml of water and supplemented with 1,000 ppm of sugar (glucose or arabinose) and placed in 300-ml plexiglass cylinders. The quantity of glucose-U-14C (sp act 360 μCi/μmol) mixed with inactive glucose was 0.17%. This was found optimal for measurement of 14CO2 production rates down to 2% of the total net production. Samples were incubated at 20°C for 48 or 68 h.

RESULTS

Analysis of the priming action. The application of glucose or other readily degradable substrates leads to a measurable increase in the CO2 production of soil (Fig. 4). After subtraction of the basal respiration (i.e., soil without supplement), the CO2 net production is obtained. The net production curve consists of CO2 produced by the oxidation of glucose and that caused by the priming action of glucose. The measurement of the exact quantity of CO2 evolved from 14C-labeled glucose allows separation of the CO2 contributed by each source. For the experiment shown in Fig. 4, a balance sheet was prepared (Table 1).

Table 1 shows that, from the organic material of the soil (6.50 g of C), a total of 0.026% was degraded to CO2 due to the priming action of the glucose.

Since the total supplement of glucose amounted to 12.0 × 10⁻⁴ g of C, the quantity of glucose assimilated by the microorganisms during the experiment was 7.8 × 10⁻⁴ g, or approximately 65%.

Besides giving quantitative data, this experiment indicates that difference curves, which are frequently used in soil respiration studies, can lead to false interpretation of results. In Fig. 4, the net production curve constructed for the glucose-supplemented soil indicates that degradation of glucose begins at 40 h and reaches its maximum at approximately 50 h. The curve derived from measurement of 14CO2, which could only have come from the glucose, indicates that glucose was degraded throughout the entire experiment and that the actual maximum of degradation occurred at approximately 46 h.

Analysis of substrate mixtures. The simultaneous degradation of substrate mixtures in soil often results in respiration curves with multiple or shouldered peaks due to the time difference in enzyme inductions. In Fig. 5 and Table 2, results from a model experiment using a mixture of glucose (1,000 ppm) and arabinose

![Fig. 4. Comparison of production rates of CO2 carbon when measured as 14CO2 (O) or calculated (●) from the difference between glucose-supplemented (□) and nonsupplemented soil (△).](http://aem.asm.org/)
(1,000 ppm) are given. The hourly production rates, which were handled by the same scheme as in Table 2, resulted in distinct maximum curves for oxidation of glucose and arabinose (Fig. 5). To relate the calculated CO₂ curves to the degradation sequence of the substrates requires that the timing of evolution of ¹⁴CO₂ from glucose-U-¹⁴C be followed. In the above example glucose was oxidized before arabinose. The quantity of glucose oxidized, as measured by inactive CO₂ (4.6 × 10⁻² g of C) and radioactive CO₂ (4.2 × 10⁻² g of C), corresponded satisfactorily.

**Analysis of inhibitory effects.** When investigating the effects of inhibitors (pesticides, etc.) on substrate degradation in soil, a simultaneous degradation of substrate and inhibitor can occur. To follow degradation of either or both of the components, the information obtained from the CO₂ production curves and the difference curves derived from these must be supplemented by the simultaneous measurement of ¹⁴CO₂ from one of the two components in the mixture.

In one experiment in which ¹⁴C-glucose or ¹⁴C-glucose plus benomyl [500 ppm active ingredient of methyl-1-(butilcarbamoyl)-2-benzimidazol carbamat] were added to soil, the appearance of maximum CO₂ production was delayed by 10 h in the presence of the inhibitor. When difference curves were analyzed, an excess of CO₂ in the Benomyl-treated sample was noted. The appearance of the extra carbon was confirmed by comparison with measurements of the total ¹⁴C produced. Both methods showed good agreement in indicating the degradation of the fungicide (Table 3).

Of the 8.7 × 10⁻² g of Benomyl carbon added, only 3.8% was oxidized to CO₂. Without glucose, however, only 2.4% of the added Benomyl carbon appeared as CO₂. The priming effect of glucose on Benomyl was therefore noticeable.

It is also of interest that, under the influence of the fungicide (Fig. 6), the ¹⁴CO₂ respiration curve showed two peaks. In the first phase (±21–33 h), the oxidation of glucose was reduced by approximately 34%, which roughly indicated the portion of Benomyl-sensitive fungi in the soil. The second phase (±26–48 h) probably indicated the development of Benomyl-tolerant organisms. The soil used in these experiments contained a high percentage of Benomyl-resistant *Mortierella* species.

**DISCUSSION**

Trapping the ¹⁴CO₂ evolved from soil during a series of 50-min incubation intervals and measuring the trapped activity in a scintillation counter result in a sensitive system (3.3 × 10⁻⁸ μCi per m³ per 100 counts per min), which is approximately three orders of magnitude higher than measurements with proportional gas tube counters or ionization chamber-type of instruments.

The combination of a commercially available CO₂ analyzer with a trapping and a collecting device for ¹⁴CO₂ allows a simultaneous and stepwise measurement of respiratory rates. The sensitivity of inactive CO₂ measurement ranges from 0.001 to 0.5% (vol/vol) CO₂.

The quantity of soil used in these experiments

**Table 2. Calculated separation of CO₂ derived from glucose and arabinose in soil between 0 and 88 h**

| Determinations                        | Carbon (g x 10⁻³) |
|---------------------------------------|------------------|
| Total C after supplement with glucose + arabinose | 42.7             |
| Total C after supplement with arabinose | -38.1            |
| C from glucose oxidation               | 4.6              |
| Total C after supplement with glucose + arabinose | 42.7             |
| Total C from soil without supplement | -31.2            |
| C from glucose + arabinose + priming action | 11.5             |
| C from glucose + arabinose oxidation only | -10.1            |
| Priming action                         | 1.4              |

**Table 3. Comparison of carbon derived from glucose and benomyl using difference curves and ¹⁴C measurement in a 48-h experiment**

| Determinations                        | Carbon (g x 10⁻³) |
|---------------------------------------|------------------|
|                                      | Difference curves | ¹⁴C measurement |
| C from glucose oxidation              | 3.4              | 3.4             |
| C from benomyl oxidation              | 0.3              | 0.3             |

**Fig. 6. Resolution of a double-peak ¹⁴CO₂-production curve, derived from Benomyl-treated soil, into phases of sensitive (Δ) and tolerant (Ω) organisms. ¹⁴C-glucose-supplemented control (○).**
is appropriate in view of the heterogeneity of soil; it is relatively large, with respect to the quantity and cost of labeled substrates. It is, however, of advantage that most preliminary experiments can be carried out under identical conditions with unlabeled substrates.

In the examples shown above, air-dried soil was chosen because the level of CO₂ liberated from dried and remoistened soil is initially low. The use of biologically active and naturally moist soil supplemented with radioactive carbon requires that precautions be taken against the uncontrolled release of CO₂.

It should be pointed out that, with the exception of the control unit, all other components of the machine can be used independently for other purposes.

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