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Metabolic Efficiency and Turnover of Soil Microbial Communities in Biodegradation Tests†

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Biodegradability screening tests of soil commonly measure $^{14}CO_2$ evolution from radiolabeled test compounds, and glucose has often served as a positive control. When constant amounts of radiolabel were added to soil in combination with increasing amounts of unlabeled substrates, glucose and some related hexoses behaved in an anomalous manner. In contrast to that of formate, benzoate, *n*-hexadecane, or bis(2-ethylhexyl) phthalate, dilution of glucose radiocarbon with unlabeled glucose increased rather than decreased the rate and extent of $^{14}CO_2$ evolution. [^{14}C]glucose incorporation into biomass and V_{max} values were consistent with the interpretation that application of relatively high concentrations of glucose to soil shifts the balance of the soil microbial community from the autochthonous (humus-degrading) to the zymogeneous (opportunistic) segment. The higher growth and turnover rates that define zymogeneous microorganisms, combined with a lower level of carbon incorporation into their biomass, result in the evolution of disproportionate percentages of $^{14}CO_2$. When used as positive controls, glucose and related hexoses may raise the expectations for percent $^{14}CO_2$ evolution to levels that are not realistic for other biodegradable compounds.

The relationship between substrate carbon assimilation and oxidation to CO_2 is referred to as yield (*Y*) or metabolic efficiency (9). It is determined by the energy content of the substrate, by the biochemical pathways of the degrading microorganisms, and by other growth conditions. During the aerobic metabolism of most substrates, *Y* approaches 0.5, meaning that one substrate carbon atom is assimilated for each carbon atom converted to CO_2.

In soil-based biodegradability screening tests, $^{14}CO_2$ evolved from radiolabeled test substrates is often used as a measure of biodegradation (6, 13). A 50% conversion of substrate carbon to CO_2 is interpreted as complete or near-complete biodegradation. The remaining biomass-incorporated ^{14}C is released slowly from humus-degrading autochthonous microorganisms and faster from zymogeneous (opportunistic) microorganisms (4).

While examining some parameters of biodegradability screening (10), we applied to soil constant amounts of radiolabel in combination with increasing amounts of corresponding unlabeled substrates. With some substrates, $^{14}CO_2$ evolution rates increased instead of decreasing with the dilution of the radiolabel. To explain this perplexing result, we developed the theory that very low concentrations of such substrates are metabolized by the autochthonous microbial community without causing significant growth or change in community composition. In this case, metabolic efficiency remains high and turnover remains low, resulting in less $^{14}CO_2$ evolution. When high concentrations of certain substrates are applied, these are metabolized principally by fast-growing zymogeneous microorganisms with lower metabolic efficiency. Being unable to use humic substances, many of these microorganisms die after the substrate is exhausted, resulting in high biomass turnover. The net result is a higher rate and greater extent of $^{14}CO_2$ evolution. The outlined scenario is of ecological interest and also has practical implications for biodegradability testing. The work described here compared our experimental results to the outlined theory.

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MATERIALS AND METHODS

Test compounds. The radiochemicals used are listed in Table 1. The unlabeled substrates used for their dilution were of the highest purity available and were obtained from Aldrich (Milwaukee, Wis.) and Sigma (St. Louis, Mo.).

Soils. The experiments described in this report were conducted with Nixon sandy loam (sand, 50%; silt, 21%; clay, 29% [organic matter, 5%] [pH 5.5 to 6.0]; water holding capacity, 0.65 g/g of dry soil) which was freshly collected and sieved (2-mm openings) in a semimoist state. At least 5 days prior to its use, the soil was adjusted close to neutrality with $CaCO_3$ in accordance with a liming curve. To avoid any nutrient imbalance, 0.25 mg of $(NH_4)_2HPO_4$ per g of soil was added as fertilizer and the moisture level of the soil was adjusted to 60% of the water-holding capacity. Characterization of the soil was performed as described by Pramer and Schmidt (8). Soil handling precautions were observed (7). As a comparison, experiments with glucose were also conducted with Lakewood sand (sand, 90%; silt, 4%; clay, 6% [organic matter, 4.9%] [pH 4.0]) and with a Nixon sandy loam sample under forest cover with high organic matter content (sand, 59%; silt, 28%; clay, 13% [organic matter, 13%] [pH 5.0]).

Test compound application and incubation. Test substrate concentrations were normalized on the basis of carbon content (11). Water-soluble substrates plus the fertilizer were applied dissolved in the water used to moisten the soil. Hydrophobic substrates were applied in 0.5 ml of *n*-hexane to 2.0 g of air-dried soil. After evaporation of the solvent, the treated soil was mixed with fresh soil to obtain a total weight of 25 g. In such cases, control samples were similarly treated with pure *n*-hexane. All concentrations were calculated on the basis of oven-dried (100°C) soil.

Incubation and measurement procedures. Soil samples (25 g) were incubated in biometer flasks (5) at 27°C. Unless noted otherwise, all measurements were done in triplicate. Standard deviations were calculated, but the error bars were smaller than the symbols. Of the alkali in the side arms (10 ml), 1 ml was placed in 10 ml of Scintiverse BD (Fisher, Springfield, N.J.) scintillation fluid and counted in a Beta-Trac 6895 instrument. Counts were corrected for background and for efficiency by the external standard ratio method. Counts were plotted cumulatively with time as percentages of the total radioactivity added. Normally, all samples received the same amount of radioactivity and the total substrate concentration was varied by dilution with unlabeled substrate. However, in one control experiment, the total glucose C concentration was fixed at 400 μg/g of soil while the normal amount of radiolabel was arbitrarily designated 5× and compared to a lower 1× level and a higher 10× level. No replicates were used in this experiment. Total CO_2 evolution and net CO_2 evolution were also measured by using the residual 9 ml of the alkali (11), but in this investigation they were used only to confirm the biological activity of the soil and the total substrate concentrations applied. They were unnecessary for the calculations presented in this report, and these results have been omitted to save space.

TABLE 1. ^{14}C-labeled compounds used in this study

Compound[a]	Source	Sp act (mCi/mmol)	Amt used (dpm/25 g of soil)
UL D-glucose	Amersham[b]	292	250,000
n-1-Hexadecane	Amersham	61	220,000
Formate	Amersham	52	150,000
UL fructose	American Radiolabeled Chemicals[c]	300	310,000
UL inositol	American Radiolabeled Chemicals	250	150,000
Ring-UL benzoate	Sigma	15	270,000
Ring-UL bis(2-ethylhexyl) phthalate	Sigma	9.8	400,000

[a] Radiochemical purity, as determined by the manufacturers by high-pressure liquid chromatography, was 98% or higher, and the compounds were used without further purification. UL, uniformly labeled. *n*-1-Hexadecane was labeled at the C-1 position only; bis(2-ethylhexyl) phthalate and benzoate were labeled at the benzene rings only.
[b] Elk Grove, Ill.
[c] St. Louis, Mo.

Calculation of the concentration effect (*Rc* value). To quantify the effect of substrate concentrations on their mineralization rate in soil, the percent radiolabel mineralized with time at a given substrate concentration was divided by the percent substrate mineralized with time at the lowest substrate concentration (0.64 to 213.5 ng/g of soil) that the specific activity of the radiolabeled substrate allowed. In relation to the total substrate concentration, the concentrations of the labeled substrates were negligible (0.02% maximal, but usually much less). *Rc* was calculated by the equation *Rc* = % $^{14}CO_2$ evolution from labeled substrate + unlabeled substrate/% $^{14}CO_2$ evolution from labeled substrate.

Extraction of soil biomass carbon. Radiolabeled biomass carbon was extracted from treated soil by the method of Sparling and West (12). In brief, replicate soil samples were fumigated with chloroform or left untreated. Subsequently, both samples were extracted with 0.5 M K_2SO_4 and the radioactivity in the extracts was measured. The radioactivity extracted from the fumigated soil minus that from the unfumigated soil represents the extracted portion of the soil biomass radiocarbon. Efficiency of radiocarbon extraction from biomass, calculated from the $^{14}CO_2$ evolution and the radiocarbon left in the soil after extraction as determined by wet combustion (1), was found to be 45 to 46%. This calculation did not take into account any substrate carbon humified.

Determination of apparent V_{max}. Three 25-g soil samples to which 0, 4, and 400 μg of glucose C per g of soil were added were incubated for 7 days. Each soil sample was suspended in 275 ml of mineral medium (3) diluted to 10% strength and agitated for 10 min at 150 rpm on a rotary shaker. The suspension was allowed to stand for 2 min to settle coarse sand particles, and the supernatant was decanted. While stirring, 9-ml aliquots of this soil suspension were distributed to 150-ml serum bottles, each containing a small test tube with 1 ml of 1 N KOH. Replicate bottles were supplied with increasing final concentrations (2, 4, 10, 40, and 200 μg/ml) of glucose C (added in 1 ml of mineral medium [total volume, 10 ml]) sealed, and incubated with shaking (150 rpm) at 27°C for 3, 6, and 12 h. Only one bottle was analyzed at each concentration and at each time point. Glucose mineralization was stopped by injecting 1 ml of concentrated H_2SO_4. After 12 h, the KOH tube was removed and the trapped $^{14}CO_2$ was counted. The data were fitted to the Michaelis-Menten equation by means of the nonlinear least-squares regression feature of Sigma Plot 4.0 for DOS (Jandel Scientific, Corte Madera, Calif.). Input estimates to the regression were obtained by inspection of the data.

Determination of the change in *Rc* values with time after treatment of soil with glucose. Replicate soil samples (25 g) in biometer flasks were either left untreated or treated with 400 μg of unlabeled glucose C per g of soil. At time zero and at appropriate time intervals thereafter, one flask of each type was treated with 1 ng of [^{14}C]glucose per g of soil, and for the next 2-days, $^{14}CO_2$ evolutions were measured and compared. The *Rc* values determined for the preceding 2 days were plotted to show their shift with the time elapsed after the pretreatment of the soil with 400 μg of glucose C per g of soil.

RESULTS

When soil was treated with formate concentrations ranging from 0.64 ng to 400 μg of formate C per g of soil, each containing the same amount of radiolabel, $^{14}CO_2$ evolution rates (Fig. 1A) and *Rc* values (Fig. 1B) showed the expected pattern. The $^{14}CO_2$ evolution rates were inversely correlated with unlabeled substrate concentrations. Since formate was mineralized very rapidly and extensively, *Rc* values converged on and stayed near 1.0 after day 3 of the experiment. In contrast, $^{14}CO_2$ evolution from glucose correlated positively with the total glucose concentration (Fig. 2A). The curves were biphasic, reflecting glucose mineralization up to around day 5, and thereafter reflected mineralization (turnover) of the ^{14}C-labeled biomass. Under identical incubation conditions, glucose became undetectable after 5 days (10). The *Rc* values stayed between 1.3 and 1.1 during the 2-week incubation period (Fig. 2B). When glucose C was fixed at 400 μg/g of soil but radiolabel addition was varied 10-fold, all samples evolved the same percentage of $^{14}CO_2$ and the *Rc* values of samples did not differ (data not shown). When the *Rc* values of glucose were

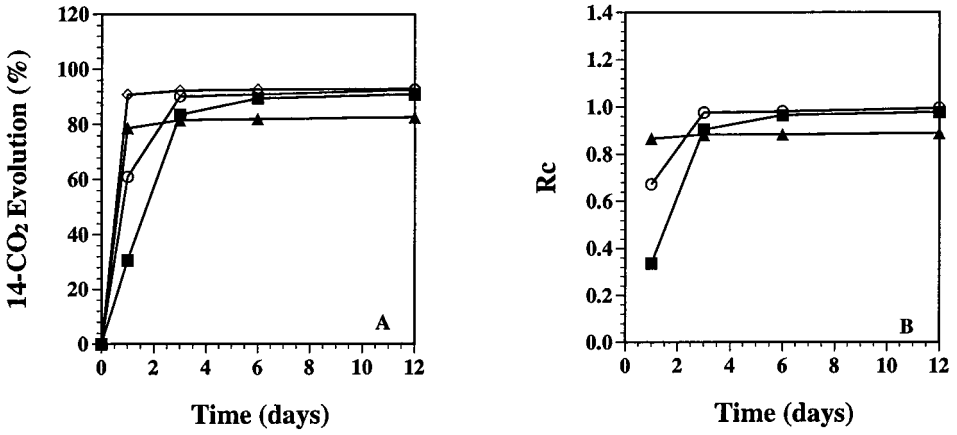

FIG. 1. Evolution of $^{14}CO_2$ from soil spiked with formate C at 0.6 ng (◇), 100 μg (▲), 200 μg (○), and 400 μg (■) per g of soil but equal amounts (6,000 dpm/g) of radiolabel. (B) Corresponding *Rc* values.

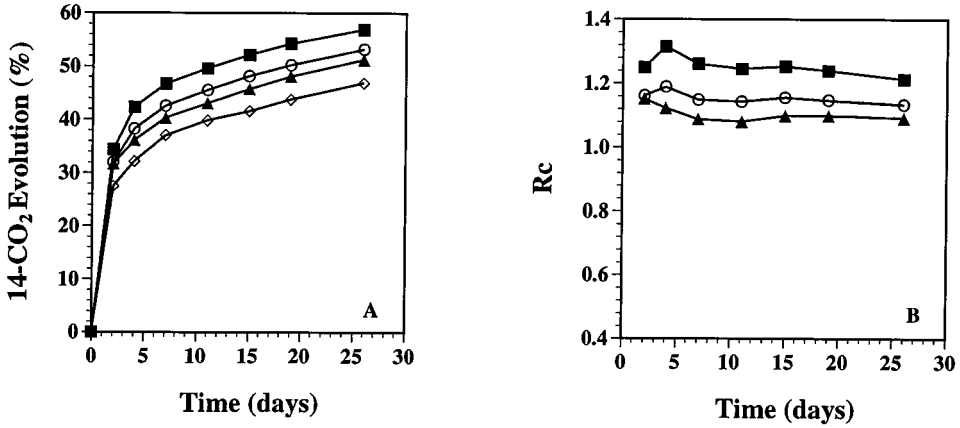

FIG. 2. (A) Evolution of $^{14}CO_2$ from soil spiked with glucose C at 1 ng (◇), 40 μg (▲), 200 μg (○), and 400 μg (■) per g of soil but equal amounts (10,000 dpm/g) of radiolabel. (B) Corresponding *Rc* values.

measured over a wide concentration range and plotted against the glucose concentration (Fig. 3), the *Rc* value equalled 1.0 at 1 μg of glucose C per g of soil. Below this concentration, *Rc* values were lower, and above it, they exceeded 1.0. These *Rc* values were calculated from several separate experiments, yet they followed a clear line of regression.

When soil samples were incubated with 0.4 and 400 μg of glucose C per g of soil, respectively, for 14 days and then fumigated and biomass carbon was extracted (12), less radiocarbon was found to be associated with the biomass of soil that was treated with the high rather than the low glucose concentration (Table 2), balancing the reverse $^{14}CO_2$ evolution patterns during the incubation period. The overall radiocarbon recoveries were reasonable, ranging from 90 to 107%.

In suspensions prepared from soil samples treated 7 days earlier with 0.4 and 400 μg of glucose C per g of soil and subsequently supplemented with increasing concentrations of radiolabeled glucose, $^{14}CO_2$ production was measured during a 12-h experiment (data not shown). The rate measurements were then used to calculate, by least-squares nonlinear regression analysis, the Michaelis-Menten constants and maximal velocities (V_{max}). These were also used to calculate the fitted curves shown in Fig. 4. Without glucose pretreatment, the apparent V_{max} of the soil was 1.98 ± 0.23 μg of glucose C per g of soil per h. Pretreatment with the low concentration of 4 μg of glucose C per g of soil left the apparent V_{max} virtually unchanged at 1.83 ± 0.21 μg of glucose C per g of soil per h. However, pretreatment with 400 μg of glucose C per g of soil tripled the apparent V_{max} to 6.01 ± 0.37 μg of glucose C per g of soil per h. The apparent K_m values derived from the plot had high standard deviations. They did not differ from each other statistically significantly, and therefore no conclusions were based on them.

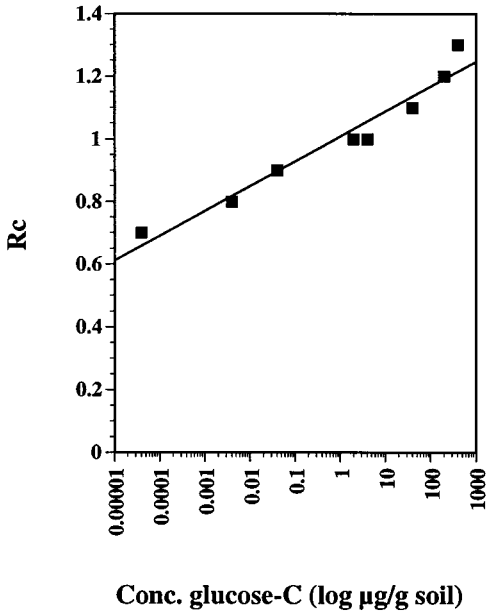

FIG. 3. *Rc* values plotted against the log of the glucose C concentration.

Figure 5 shows the temporal changes in *Rc* after treatment with 400 μg of glucose C per g of soil. The control soil received no glucose pretreatment. Each datum point represents the *Rc* value calculated for the preceding 2-day interval. During these periods, newly applied 1-ng glucose radiocarbon spikes per g of soil were mineralized in both the pretreated and control soil samples. The plotted *Rc* values reflect the differences between the pretreated and control soil samples and their shift with time. The first *Rc* value, representing the 0- to 2-day period, was already elevated to 1.23. The second point, representing the 1- to 3-day period, was even higher at 1.33. Thereafter, the *Rc* values declined gradually. It needs to be noted that the *Rc* values in Fig. 5 were measured in replicate soil samples for successive 2-day periods, while the *Rc* value in Fig. 2B was plotted cumulatively with time in the same sample. Consequently, the two curves should and do differ in shape.

TABLE 2. Radiocarbon distribution 14 days after soil treatment with two concentrations of glucose, each containing the same amount of radiolabel

Treatment with glucose (μg of C/g of soil)	$^{14}CO_2$ evolved (%)	K_2SO_4 flush (%)	^{14}C left in soil (%)[a]	Balance of ^{14}C (%)
0.004	31.1	33.7[b]	42.0[b]	106.8
0.004	31.1	4.7[c]	54.3[c]	90.1
400.0	52.2	23.4[b]	30.6[b]	106.2
400.0	52.2	2.4[c]	40.9[c]	95.5

[a] Determined after wet combustion.
[b] After chloroform fumigation.
[c] Without chloroform fumigation.

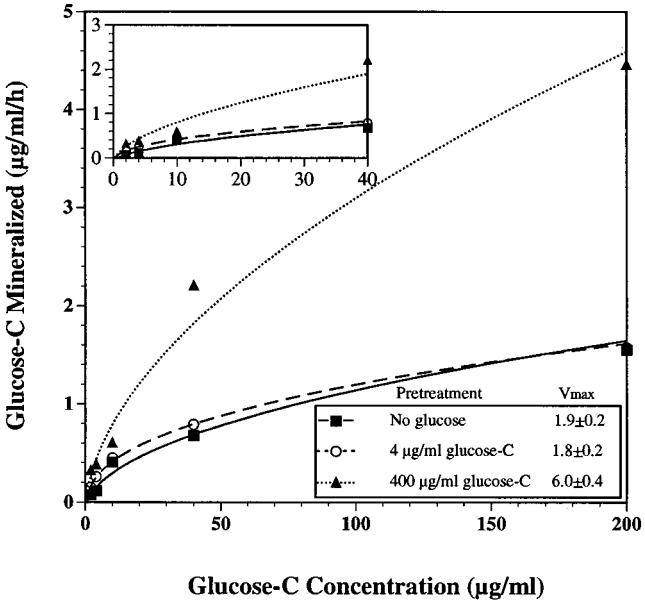

FIG. 4. Michaelis-Menten plots of glucose mineralization in soil slurries prepared from soil samples treated 7 days earlier with no glucose or 4 or 400 μg of glucose C per g of soil. The soil slurries (10 ml) were incubated with 2, 4, 10, 40, and 200 μg of radiolabeled glucose C per ml for 3, 6, and 12 h. The $^{14}CO_2$ evolved was trapped and counted. For better readability, the 0- to 50-μg/ml portion of the plot is expanded below. V_{max} is expressed as micrograms of glucose C per milliliter per hour.

Table 3 summarizes and classifies the behavior of all of the substrates we tested in soil in terms of their concentration effects, without presenting the results graphically. The substrates we tested fell into two groups. Group I [benzoate, formate, *n*-hexadecane, and bis(2-ethylhexyl) phthalate] exhibited the predictable behavior illustrated for formate in Fig. 1. For these compounds, *Rc* values were below 1.0 during the early days of the experiments. The *Rc* values of the rapidly degrading water-soluble substrates (benzoate and formate) returned to near 1.0 rapidly. With the slowly degrading hydrophobic substrates [hexadecane and bis(2-ethylhexyl) phthalate], this return was more gradual. For these reasons, the typical *Rc* values of the former compounds are shown for day 1 and those for the latter compounds are shown for day 5. The behavior of the compounds in group II (fructose, glucose, and inositol) was like that of glucose in Fig. 2. They attained *Rc* values above 1.0 early in the experiment and maintained them at this level for long periods. Group II *Rc* values are also listed for day 5.

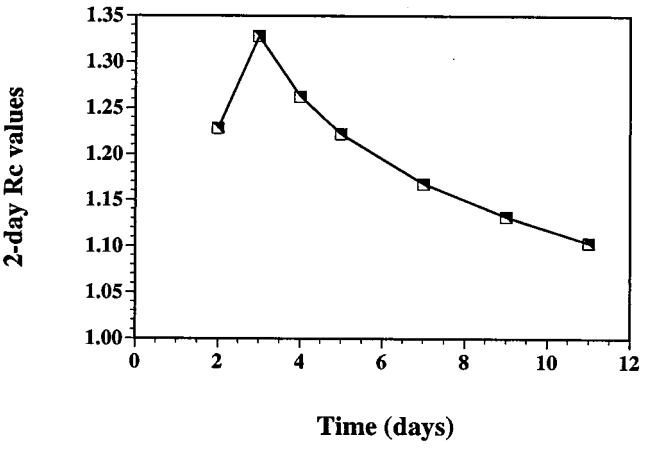

FIG. 5. Change of *Rc* values with time in replicate soil samples that were either left untreated or treated with 400 μg of unlabeled glucose C per g of soil. At successive time points (days 0, 1, 2, 3, etc.), one treated flask and one untreated flask were supplemented with 1 ng of radiolabeled glucose C per g of soil. The evolution of $^{14}CO_2$ was measured for the 2-day interval preceding the plotted *Rc* value.

TABLE 3. Comparison of the concentration effects of the substrates tested in soil

Substrate[a]	*Rc*	Day	Group
Benzoate	0.27	1	I
Formate	0.35	1	I
Hexadecane	0.57	5	I
Bis(2-ethylhexyl) phthalate	0.16	5	I
Fructose	1.5	5	II
Glucose	1.4	5	II
Inositol	1.7	5	II

[a] All were tested at 400 μg of substrate carbon per g of soil.

Workload considerations restricted us to conducting most of our experiments with one soil type. However, we wanted to ascertain that the observed concentration effect of glucose was a general phenomenon and not the characteristic of a single soil. Therefore, the type of experiment shown in Fig. 2 was repeated with Lakewood sand and a forest-covered high-organic-matter (13%) Nixon sandy loam sample. In both soil samples, glucose had a positive concentration effect, with *Rc* values of 1.20 and 1.19, respectively (data not shown).

DISCUSSION

Kinetic considerations and availability. The mineralization rates of substrates [*S*] in soil may be described by the familiar Michaelis-Menten equation (14) $dCO_2/dt = k[S]/K_m + [S]$. However, our experiments measured $^{14}CO_2$ rather than total CO_2 evolution from the added substrate. The amount of added radiolabel (counts per minute) was constant for one substrate, and specific activity was diluted by increasing concentrations of unlabeled substrate carbon (counts per minute per [*S*]). At very low substrate concentrations, the increases of the reaction rates due to increased [*S*] compensate for the dilution of radiocarbon. However, as saturating concentrations are approached, the $^{14}CO_2$ evolution rates are expected to decline as [*S*] increases in relation to counts per minute (cpm): $d^{14}CO_2/dt = (\text{cpm}/[S])(k[S]/K_m + [S])$.

The expected decline was observed with the class I substrates (Fig. 1 and Table 3), but class II substrates, such as glucose (Fig. 2) and the structurally related hexoses fructose and inositol, showed the opposite behavior. With increasing dilution of the radiolabel, the rate and extent of $^{14}CO_2$ evolution increased, resulting in *Rc* values above 1. The explanation for this phenomenon is unclear. We have considered the possibility that at very low concentration levels, substrates may become partially unavailable because of absorption. However, it is difficult to rationalize why glucose would be affected in this manner when the other substrates, some polar and some hydrophobic, remained available under the same conditions. When glucose C was kept at 400 μg/g of soil but the amount of radiolabel added was varied 10-fold, there was no difference in the percentages of $^{14}CO_2$ evolution or in the *Rc* values (data not shown). This control experiment ruled out possible experimental artifacts.

Metabolic efficiency and turnover rates. In a heterogeneous microbial community, low substrate concentrations select for microorganisms with intrinsically high yield coefficients, while high substrate concentrations select for microorganisms with high growth rates with little or no selective pressure for high yield coefficients (2). We interpret the data in Table 2 as evidence of such selection. Efficiency of biomass extraction, calculated as described by Sparling and West (12), was 45 to 46%, regardless of pretreatments. Consequently, at the low glucose concentration, *Y* was 0.66; at the high glucose concentration, *Y* was only 0.47. Biomass plus CO_2 left 5 and 2% of the radioactivity unaccounted for: these are the amounts extracted by K_2SO_4 from the two unfumigated-soil controls. Whatever these radioactivities represent, they are rather small amounts and cannot change the calculated *Y* values significantly.

The results in Fig. 4 show that pretreatment with relatively high levels of glucose C (400 μg/g) tripled the V_{max} of the soil microbial community for glucose metabolism. This could represent a shift to new glucose-degrading populations with faster enzymes but may also be caused by a simple increase in the original population, making more enzyme available. While Fig. 4 indicates that a change in the population of glucose degraders occurs, it does not specify whether the change is an increase, a substitution, or a combination of the two. The temporal change in the *Rc* values (Fig. 5) shows that *Rc* peaks between days 2 and 4 after the application of 400 μg of glucose C per g of soil. Previous work showed that the same amount of glucose under the same incubation conditions became analytically undetectable by day 5 (10). Up to that time, the *Rc* is likely to reflect a direct interaction of glucose remaining from the pretreatment with the low concentration of radiolabeled glucose added later. Past day 5, the declining but still elevated *Rc* values can no longer reflect direct substrate interactions. Instead, we suggest that these *Rc* values reflect the more rapid turnover of the zymogeneous (opportunistic) microbial population built up by the glucose pretreatment. As this segment of the soil microbial community dies off or becomes dormant, the *Rc* effect eventually disappears.

***Rc* and substrate types.** With the substrates tested, *Rc* values that significantly exceeded 1 were produced only by glucose and closely related hexoses. One may rationalize that hydrophobic substrates are utilized too slowly and formate has too low an energy content to cause population shifts as dramatic as those caused by glucose and related compounds. However, benzoate is water soluble, has a high energy content, and is utilized as rapidly as glucose (11), yet in repeated experiments, it failed to produce *Rc* values comparable to those produced by glucose and the other two hexoses (Table 3). It is possible that benzoate is used by a much narrower segment of the soil microbial community than is glucose. If so, these microorganisms would merely multiply but would not be replaced by different populations with lower yield coefficients and higher turnover rates. Although there is no obvious relationship to *Rc*, the behavior of glucose in soil is also unusual insofar as it causes a strong and sustained increase in the biodegradation of the soil organic matter (11). The peculiar behavior of glucose and related hexoses suggests that they should not be used as positive controls in biodegradation tests in either unlabeled or radiolabeled form.

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