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Testing of Some Assumptions about Biodegradability in Soil as Measured by Carbon Dioxide Evolution†

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Conversion to CO_2 upon incubation in aerobic soil is one of the standard test procedures to assess biodegradability. It may be measured with unlabeled test compounds in biometer flasks. In this case, the background CO_2 evolution by unamended soil is subtracted from the CO_2 evolution by the amended soil and the resulting net CO_2 evolution becomes the measure of biodegradation. Alternately, $^{14}CO_2$ release from radiocarbon substrates is measured to assess biodegradability. Both approaches measure ultimate (complete) biodegradation and bypass the theoretical and technical limitations of residue analysis. This report examines the underlying assumptions that, except for carbon content, conversion percentage to CO_2 is relatively independent of chemical composition, that CO_2 production is proportional to the amount of added test compound, and that the background CO_2 evolution of the soil is not influenced by the test substance. Work with unlabeled and radiolabeled substrates proved the first two assumptions to be essentially correct. However, more than half of net CO_2 production may represent the mineralization of biomass and soil organic matter, some of it unrelated to the test compound. The soil microbial community in its nongrowing steady state appears to convert a much lower percentage of a radiocarbon substrate to $^{14}CO_2$ than a growing soil community that responds to a substantial substrate addition. These findings may help to improve test methods and may aid in the interpretation of test results.

The Toxic Substances Control Act calls for a premanufacture review of novel chemical substances concerning their environmental fate and effects, including biodegradability (10). The methodology of testing is in continuous evolution, but first-tier tests for aerobic biodegradation in soil normally involve CO_2 evolution studies from nonlabeled or from ^{14}C-labeled materials. Test protocols, as exemplified by the U.S. Food and Drug Administration (FDA) Environmental Assessment Technical Assistance Handbook (11) evolved from techniques used in biodegradation studies on pesticides and chlorobenzenes, respectively (4, 7). They were selected as standard procedures because of their relative simplicity and broad applicability and appear to be satisfactory in these respects. Nevertheless, they were originally developed for a more limited purpose and did not undergo the background investigations that broadly applied standard techniques deserve. The work described here attempts to provide some of this missing yet necessary background information. The current FDA test protocol assumes an organic compound to be biodegradable if during the test period more than 50% of its carbon content is released as CO_2. The work performed here attempts to answer the following specific questions: (i) Is the percent conversion to CO_2 of a biodegradable compound influenced by its chemical composition other than carbon content? (ii) Is the percent conversion to CO_2 of a test compound influenced by the concentration applied to soil? (iii) Does net CO_2 evolution represent actual substrate mineralization as reflected by $^{14}CO_2$ evolution?

MATERIALS AND METHODS

Test compounds and soil. Test materials were chosen to be highly biodegradable, of low volatility, and to represent a variety of chemical structures. Unless specified otherwise, glucose (carbohydrate), adipic acid (aliphatic diacid), benzoic acid (aromatic acid), and *n*-hexadecane (aliphatic hydrocarbon) were applied to soil at 1 mg/g of soil (dry weight basis). These chemicals were obtained from Fisher Scientific Co. (Springfield, N.J.) and were of certified reagent grade (over 99% pure). The acids were neutralized with NaOH prior to use [U-^{14}C]glucose, specific activity of 10.6 GBq/mmol and purity of 99.6%, determined by high-pressure liquid chromatography by the manufacturer, was obtained from Amersham Corp. (Arlington Heights, Ill.). The delivered solution was diluted either with water or with unlabeled glucose solution to obtain the desired dose per biometer flask. This dose was then added as part of the liquid used in adjusting the soil moisture level.

Biometer flasks (4) contained the equivalent of 25 g of dry soil each. Freshly collected Nixon sandy loam (texture: 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) (3) was used in all experiments. The soil samples were collected between May and August, from the same location. The plant cover of the soil was landscape lawn. To optimize biodegradation conditions for the tests, we raised the original pH of the soil to 7.5 by addition of 10 mg of $CaCO_3$ per g of soil. This was done at least 5 days prior to the biodegradation tests to avoid the release of neutralization-derived CO_2 in the biometers. To avoid any limitation of the biodegradation process by mineral nutrient deficiency, we added 0.6 ml of a 1% solution of $(NH_4)_2PO_4$ to every biometer flask, plus sufficient distilled water to bring the soil moisture level to 60% of holding capacity (8). All test substrates except *n*-hexadecane were dissolved in this water. Hexadecane was applied to 1 g of air-dried soil, and this dry soil was

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thoroughly mixed with fresh soil equivalent to 24 g by dry weight. For this type of sample application, a separate control was prepared with 1 g of air-dried soil but no hexadecane. Otherwise, the control was fresh soil that was treated in every respect like the test samples but received no test compound. Incubation was at 27°C. Titrations for CO_2 and aeration of the flasks through the Ascarite filters were performed initially daily and at 2- to 3-day intervals later in the experiment.

Net CO_2 evolution, defined as the CO_2 evolution by substrate-amended soil samples minus CO_2 evolution by identical but unamended soil, was calculated by subtracting, at each measurement point, the average cumulative CO_2 evolution of the unamended soil from the average cumulative CO_2 evolution of the substrate-amended soil. To calculate standard deviation, the cumulative average CO_2 evolution values of the unamended soil samples were also subtracted from the cumulative CO_2 evolution values of the individual amended soil samples. The CO_2 evolution by unamended soil is referred to, for brevity, as background CO_2 evolution.

Analytical and radiochemical procedures. Glucose in soil was determined by the procedure of Fales (6). Anthrone reagent (Eastman Kodak Co., Rochester, N.Y.) was freshly prepared for the measurements. For a standard curve, 25-g soil samples were dosed with the appropriate concentrations of glucose. Immediately, 25 ml of water was added, and the sample was shaken for 2 min and centrifuged to obtain a clear supernatant. This was decanted, and the soil residue was washed in a similar manner with 15 ml of water. The extracts were combined and adjusted to a volume of 50 ml, and 1 ml of this extract was used for glucose determination. Incubated soil samples were extracted in an identical manner. A_{620} was measured with a Shimadzu model UV-265 spectrophotometer. Soil extract with no added glucose plus anthrone reagent served as a blank.

In experiments that measured both total CO_2 and $^{14}CO_2$ evolution, 1 ml of KOH was retrieved from the biometer side arm and placed in a counting vial with 15 ml of Oxosol (National Diagnostics, Manville, N.J.) counting solution. Thereafter, the normal biometer titration procedure was performed on the residual alkali, but the results were adjusted for the volume decrease due to radioactivity counting.

Radioactivity was measured in a liquid scintillation counter (Beta-Trac 6895; TM Analytic, Elk Grove Village, Ill.). Samples from biometer flasks without radioactive glucose were used for background correction. Counting efficiency was corrected by the external standard ratio method.

All tests were performed with triplicate biometer flasks. Standard deviation (1 SD) is indicated in form of error bars. They were omitted when smaller than the symbol in the figures.

RESULTS

Effects of chemical composition and mode of application. Figure 1 shows net CO_2 evolution from four test compounds of strongly differing chemical composition during a 30-day incubation. All these compounds were found to be highly biodegradable in preliminary tests (data not shown). As expected, net CO_2 evolution strongly depended on the carbon contents of the test compounds, but other features of chemical composition had little effect on the percentage of the carbon mineralized. About 50% of the substrate carbon was released in an initial flush of CO_2 within the first 10 days of the experiment. More-gradual mineralization followed

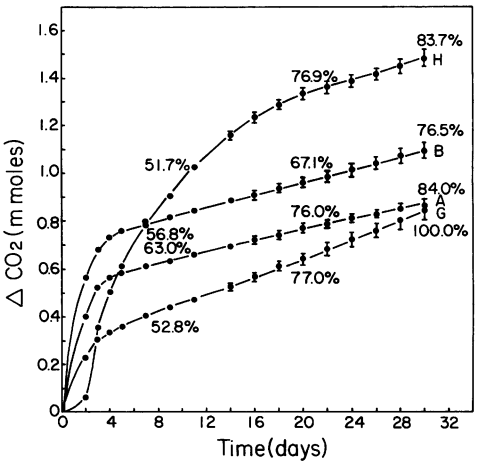

FIG. 1. Cumulative net CO_2 evolution (ΔCO_2) during incubation in soil from the substrates *n*-hexadecane (H), benzoic acid (B), adipic acid (A), and glucose (G), 1 mg/g of soil each, with 25 g of soil per biometer flask. The points represent the average of three replicates. The error bars represent 1 standard deviation (SD), omitted when smaller than the point symbol. The numbers at 9, 20, and 30 days represent the percent conversion of each substrate's calculated carbon content to CO_2.

thereafter, but the rate did not return to soil background level within the 30-day incubation. This second slope of CO_2 release was more steep for glucose than for the other test compounds, and net CO_2 from glucose reached 100% by day 30. This pattern was reproducible with glucose and closely related compounds such as starch (data not shown), but its cause needs further investigation.

Small amounts of hydrophobic liquids may form droplets in wet soil. In our experience, they are more evenly distributed with air-dry soil as a vehicle as was done here with *n*-hexadecane. It is very important in such a case to treat the soil control in an identical manner. Figure 2 compares CO_2 production by the two soil controls, one containing 1 g (4%) of air-dried soil. This treatment resulted in the release in 30 days of 0.4 mmol of excess CO_2, as much as the addition of 12 mg of glucose would produce.

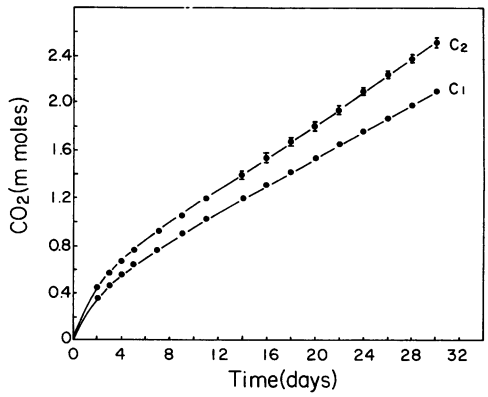

FIG. 2. Cumulative total CO_2 evolution by the normal soil control (C_1) of the experiment shown in Fig. 1. The second soil control (C_2) is similar, except 1 g of the 25-g soil samples was first air dried and then rewetted. Average of triplicates; the error bars represent 1 SD.

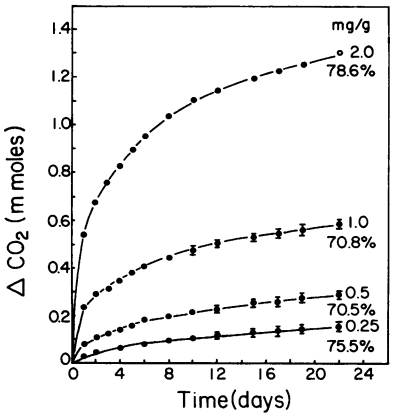

FIG. 3. Cumulative net CO_2 evolution from 25-g soil samples, amended with 0.25, 0.5, 1.0, and 2.0 mg of glucose per g of soil. Average of triplicates; error bars represent 1 SD. The numbers at day 22 represent the percent conversion of the calculated substrate carbon to CO_2.

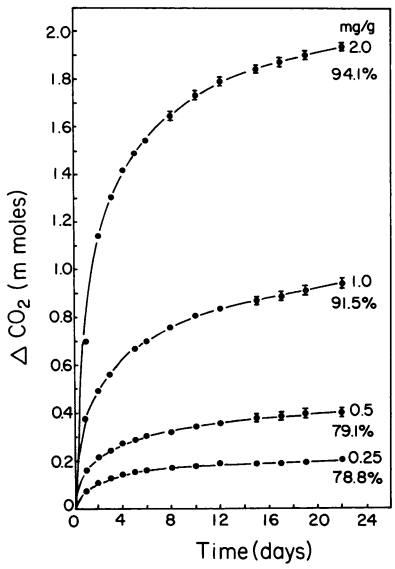

FIG. 4. Cumulative net CO_2 evolution from 25-g soil samples, amended with 0.25, 0.5, 1.0, and 2.0 mg of adipic acid per g of soil. Average of triplicate flasks. The numbers at day 22 represent the percent conversion of the calculated substrate carbon to CO_2.

A comparison of the net CO_2 values in Fig. 1 with the background CO_2 evolution of unamended soil in Fig. 2 gives an idea of the typical background noise to biodegradation measurements in soil when unlabeled substrates are used. Selecting, somewhat arbitrarily, day 20 for comparison, the cumulative background CO_2 evolution of unamended soil is 1.5 mmol, and 1.8 mmol when dry soil was added. This compares to a net cumulative CO_2 evolution of 0.65 mmol from glucose and 1.35 mmol from hexadecane, respectively, when the compounds were applied at the 1 mg/g of soil (1,000 ppm) level. This is a comfortable ratio, with the signal (net CO_2) being 43 and 75% of the background CO_2 evolution. Obviously, at lower substrate application levels, the signal-to-background ratio becomes less favorable.

Effects of test substrate concentrations. When glucose (Fig. 3) and adipate (Fig. 4) were added at levels from 0.25 to 2.0 mg/g of soil, net CO_2 evolution was essentially proportional. Considering that in this experiment the cumulative background CO_2 evolution of the control soil on day 22 was 2.028 mmol, it is obvious that the 0.5- or 1.0-mg/g substrate addition levels give more reliable results than lower additions. At less than 0.1-mg/g substrate addition level, the technique is not reliable (2), and the 0.2-mg/g addition level recommended by the FDA (11) requires very precise technique for good results.

Sources of net CO_2 evolution. In Fig. 1, net CO_2 release from glucose reached 100%, yet this soil sample continued to evolve excess CO_2 compared with the untreated soil control. This and similar observations not shown here suggested that stimulated mineralization of soil organic matter may contribute to net CO_2 evolution. Figure 5 shows the disappearance of glucose from soil, as measured chemically by the anthrone method, versus net CO_2 evolution over a 21-day incubation. Glucose was not detectable after day 4 and was presumably exhausted by day 5. This coincided with a rate change in CO_2 evolution. The secondary lower rate of excess CO_2 evolution continued linearly and reached 78% by day 21. Between days 5 and 21, excess CO_2 evolved from something other than glucose, this something other contributing at least one half of the recorded net CO_2.

Figure 6 reveals a rather unexpected relationship between $^{14}CO_2$ evolution from radiolabeled glucose and net CO_2 evolution. When radiolabeled glucose alone was applied to soil, CO_2 evolution did not change measurably compared to the soil control (curve not shown). This is understandable, since the application of 1,250,000 dpm per flask resulted in the addition of only 350 ng of glucose. In the first 5 days of the experiment, a surprisingly low amount (17%) of the radiocarbon was mineralized. Thereafter, mineralization of radiocarbon became very slow, amounting to 0.2% per day.

When radiolabeled glucose was added in combination with 1 mg of unlabeled glucose per g of soil, radiocarbon mineralization in the first 5 days increased to 26%. After that, the radiocarbon mineralization rate declined to 0.4% per day. The same experiment was conducted also at 125,000 and 350,000 dpm of radiolabeled glucose added (35 and 95 ng of glucose, respectively). The results were identical in terms of percent radiocarbon releases and are, therefore, not presented. Net CO_2 release (the difference between the 1 mg of glucose per g of soil and soil control curves) at day 5 was 0.31 mmol, and it was 0.37 mmol at day 10, representing 37.0

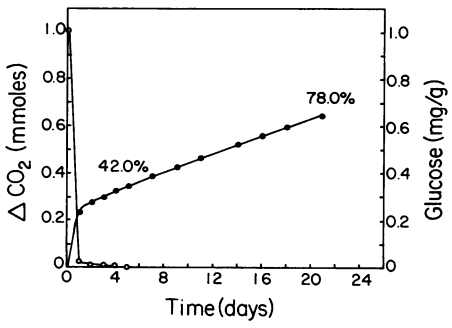

FIG. 5. Cumulative net CO_2 evolution (●) from 25-g soil samples, amended with 1.0 mg of glucose per g of soil. Average of triplicate samples; the error bars represent 1 SD. Residual glucose in soil (○) was analyzed in individual flasks daily by the anthrone method (6). The numbers at day 5 and 21 represent the percent conversion of the calculated substrate carbon to CO_2.

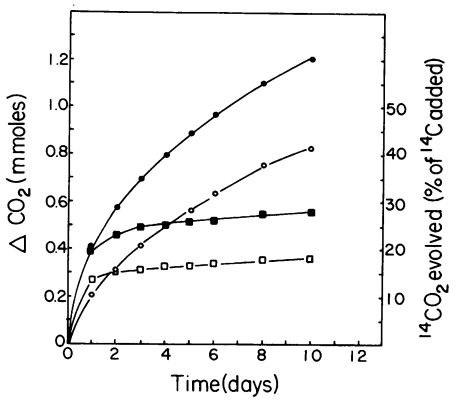

FIG. 6. Cumulative total CO_2 evolution from 25 g of soil amended by 14 ng of radiolabeled glucose (50,000 dpm) per g of soil (○). This CO_2 evolution was identical to that of the soil control (data not shown). The same amount of radiolabeled glucose was also applied in combination with 1.0 mg of unlabeled glucose per g of soil (●). Percent conversion of the radiolabel to $^{14}CO_2$ is also shown from the soil that received radiolabeled glucose only (□) and from soil that received, in addition, 1.0 mg of unlabeled glucose per g of soil (■). Average of triplicate measurements; the error bars (1 SD) are smaller than the symbols and are not shown.

and 45.2% glucose mineralization, respectively. Compared with the $^{14}CO_2$ mineralization rates, net CO_2 evolution rates after day 5 remained relatively high, amounting to 2% per day.

Additional work will be necessary to address, in a systematic way, the variables associated with the nature of test soils. While no attempt was made here to accomplish this, when the experiment shown in Fig. 6 was repeated with Nixon sandy loam from a location with a higher organic matter content (7% instead of 5%), both $^{14}CO_2$ and net CO_2 evolution percentages were found to increase (data not shown). At day 10, $^{14}CO_2$ evolution from 1 mg of glucose per g of soil (563,000 dpm per flask) was 42.5% ± 1.2%, increasing to 49.4% ± 1.3% at day 21. Net CO_2 evolution values in the same experiment were 82% ± 3.9% and 129% ± 2.5% at days 10 and 21, respectively.

DISCUSSION

Advantages of net CO_2 and $^{14}CO_2$ evolution biodegradability testing. In contrast to residue analysis, net CO_2 and $^{14}CO_2$ evolution measurements are simple, nondestructive, and measure ultimate biodegradation (mineralization) rather than a possibly limited structural change. If appropriately ^{14}C-labeled test material is available, the measurements and their interpretations are relatively straightforward. This is unfortunately balanced by the technical difficulty and expense involved in obtaining labeled material. The licensing and the waste disposal problems connected with radioactive work are also a drawback. A 1992 update of the test guidelines by the U.S. Environmental Protection Agency (5) recommends the use of radiolabeled test compounds in biometer flasks, yet the document acknowledges that labeled test compounds in some cases are impossible to obtain. Thus, some biodegradation tests with unlabeled compounds are likely to continue. Fortunately, biometer measurements show a high degree of precision and consistency. In experiments 3 to 4 weeks long, the cumulative standard deviation among triplicate flasks was a maximum of 5%, whether calculated for total or net CO_2 production, and was usually considerably less than that. In shorter experiments of 10 days, the cumulative standard deviations of total, net, or $^{14}CO_2$ evolutions in triplicate flasks were within 2% and could not be represented by error bars due to their low values. This consistency is a definite advantage for a standard testing technique. There is some variation between the microbial activity of soil samples, even when freshly collected from the same location and handled in an appropriate manner (8). These differences are quantitative rather than qualitative and result in higher or lower background CO_2 evolution, along with faster or slower net CO_2 evolution rates. During 25 years of experience with the technique, we found extensive rate variations but never positive-negative contradictions when we conducted biodegradation tests in soil samples collected in various seasons and from various locations. This observation may not apply to soils that fail to support normal plant life or are mistreated during collection or storage.

An anomalous burst of CO_2 evolution upon remoistening of air-dried soil is a well-known phenomenon and it was cautioned against earlier in connection with biodegradation tests (8). If the homogeneous application of a hydrophobic sample necessitates the air drying of some of the soil, a corresponding portion of the control soil needs to be treated identically to avoid serious errors. The burst of CO_2 represents primarily mineralization of soil biomass killed by the drying process. Physical changes caused by drying and remoistening may contribute to some desorption and greater availability of non-biomass soil organic matter.

Chemical composition other than carbon content appears to influence minimally the percentage conversion to CO_2 (Fig. 1), although glucose, the presently recommended positive control substance in biodegradation tests, appears to sustain unusually high CO_2 evolution rates long after its disappearance (Fig. 5). Conversion percentage of test compounds to CO_2 was relatively independent of concentration within the tested 0.25- to 2.0-mg/g of soil range. To achieve easily measurable net CO_2 evolution above soil background, 0.5- or 1.0-mg levels of test compound per g of soil appear to be preferable to the presently recommended 0.2 mg/g. Toxicity of a test compound in soil is rarely a problem, because reversible adsorption greatly reduces the effective concentrations to which microorganisms are exposed.

Origin of carbon in net CO_2 evolution. A somewhat disturbing aspect of our results is the fact that while CO_2 evolution seemed proportional to the carbon content and the concentration of the test substance, at least one-half of the evolved net CO_2 did not come directly from the test substance (Fig. 5). In some cases, the net CO_2 evolution may exceed 100% of the carbon added in the form of the test substance.

It has been reported that the addition of readily decomposable organic matter stimulates the mineralization of indigenous soil organic matter (1). The magnitude of this priming effect could vary from soil to soil, depending on organic matter content and on seasonal or climatological factors. However, in our tests, the responses appeared to be proportional to the added substrate carbon, and the inclusion of a positive control, as recommended in the FDA test protocol (11), should allow a correction for the variable of soil quality.

Biomass yield coefficients in aerobic microbial metabolism depend greatly on the energy content of a substrate (9), but conversion to CO_2 in soil appeared to depend on carbon content only (Fig. 1). It has been assumed that in short-term soil biodegradation studies, roughly 50% of substrate carbon

is converted to CO_2 (1, 11). In experiments of 1 month or longer, substrate carbon initially fixed in biomass is also mineralized in part. Together with substrate-stimulated mineralization of indigenous soil organic matter, this may raise the net CO_2 evolution in response to a substrate above 100% of the added substrate carbon. It remains to be explored whether this phenomenon is restricted to highly degradable substrates like the ones used in this study, or whether it applies broadly to all substrate additions.

The relationship between radiocarbon evolution and net CO_2 evolution from glucose in Fig. 6 was unexpected. The low 16 to 17% radiocarbon release from submicrogram amounts of glucose may be ascribed to unavailability due to absorption. Alternatively, the nongrowing steady-state microbial community may turn over glucose very differently from a community that is growing in response to significant substrate addition. The difference between radiocarbon mineralization and net CO_2 evolution (0.5 and 2% per day, respectively) after day 5 confirms that soil organic matter not directly related to glucose addition was part of the net CO_2 evolution response. If only glucose breakdown products and microbial biomass synthesized in response to glucose addition were mineralized, one would expect the two rates to be similar.

As a result of these investigations, we conclude that net CO_2 and $^{14}CO_2$ evolution measurements are useful as first-tier tests for assessing biodegradability in soil. Nonradiolabeled compounds should be tested in the 0.5- to 1.0-mg/g of soil range to achieve net CO_2 evolution rates that are easily distinguished from the soil background. Radiolabeled glucose and probably also other test compounds, if applied below 1 μg/g of soil, are converted to $^{14}CO_2$ in much lower percentages than test compounds applied at 1.0 mg/g of soil. Radiolabeled CO_2 originates strictly from the test substrate plus test substrate-derived biomass, but our results show that stimulated biodegradation of indigenous soil organic matter contributes to net CO_2 evolution. Consequently, for mineralization of the same amount of test substrate in soil, conversion percentages to $^{14}CO_2$ are inherently lower than net CO_2 values, when the latter are expressed as percentage of the theoretical maximum.

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