

Carbon Monoxide Metabolism in Roadside Soils

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Air-dried soils which were equilibrated under relative humidities greater than 93% or moistened with liquid water showed marked increases in their capacities to oxidize CO to CO₂. Liquid water addition in excess of saturation resulted in lower CO oxidation rates, reflecting the limited diffusion of CO through the aqueous phase. After 35 days' storage under 100% relative humidity, the capacity for CO oxidation decreased to 21% of the value observed with a freshly collected sample. Incubation of this stored soil under an atmosphere containing 200 ppm of CO (250 mg/m³) for 21 days resulted in a sevenfold increase in CO oxidation. A correlation was noted between the CO oxidative activity and the history of previous exposure of soils to high ambient levels of CO. The organisms responsible for CO oxidation apparently comprise a small fraction of the microbial population in the soils. With a roadside soil the oxidation of CO provided the driving force for the assimilation of CO₂. The stoichiometry of the oxidative and assimilatory reactions in soil was in the range of values reported from laboratory studies with CO chemoautotrophs (carboxydobacteria). It is proposed that the population and activity of CO-oxidizing microorganisms increase in response to increasing levels of CO in the environment.

In the past decade soil microorganisms have been recognized as the major biological agents for removal of CO from the lower layers of the atmosphere. From activity measurements with a variety of soils Inman et al. (8) estimated that U.S. soils could remove 5.7×10^{14} g per year. Extrapolating these data to global conditions (9), this microbiological process could remove 1.1×10^{16} g of CO per year or about ten times the total production rate from natural and anthropogenic sources. A lower potential activity of 4×10^{14} g of CO per year (worldwide) was estimated from measurements made by Seiler and co-workers (9, 15). The lower value may be more realistic from a global point of view since the calculations were made from assays employing ambient levels of CO, i.e., 0.2 to 0.5 ppm (0.25 to 0.625 mg/m³). The higher estimate was made from assays employing 100 ppm of CO, and others (12) have interpreted the resulting value to reflect potential activity in soils subjected to heavy CO pollution. From a study with field and forest soils, Heichel (3) concluded that the soil surface of the earth removes CO at a minimum annual rate equivalent to 14% of the tropospheric level. All three sets of data may be criticized on the basis that the calculations were made from assays with a limited number of samples, or that the assays may not have realistically simulated the ambient conditions.

Considering the process of CO destruction in

soil to be microbial in nature, there are two known modes of enzymatic action which result in the oxidation of CO to CO₂. The microbial action may be either specific or nonspecific. With regard to the latter, the process is catalyzed by enzymes of unknown function and importance. This process may well be an example of coincidental metabolism (cometabolism), i.e., catalysis of a reaction with a second substrate by an enzyme whose primary specificity is for another substrate (4). The aerobic and anaerobic microorganisms which catalyze this process have been described elsewhere (2, 7, 12). Of importance here is the fact that the responsible organisms oxidize CO to CO₂ and derive no apparent benefit from the reaction. Consequently, there is no reason to assume that the populations would increase in size in response to increasing CO levels.

Carbon monoxide is a carbon and energy source for the specific microflora. The energy conserved during the oxidation of CO to CO₂ drives the assimilation of CO₂ by an autotrophic process. These chemoautotrophs (chemolithotrophs) are commonly referred to as the carboxydobacteria. The stoichiometries reported for CO utilization are as follows: $24 \text{ CO} + 11 \text{ O}_2 \rightarrow 23 \text{ CO}_2 + 1 \text{ cell-C}$ (12), and $2.19 \text{ CO} + 1 \text{ O}_2 \rightarrow 1.83 \text{ CO}_2 + 0.36 \text{ cell-C}$ (11). If these findings from pure culture studies can be extrapolated to the soil ecosystem, the carboxydobacteria would not

only remove CO but would increase in numbers in response to elevated levels of the pollutant.

There is no unanimous opinion regarding the relative importance of the specific and nonspecific oxidizers in CO removal. Support for the importance of the carboxydobacteria is based on their ubiquitous distribution and "frequent" occurrence in soils subjected to CO pollution (17). However, to our knowledge there are no quantitative data to support this supposition. The enrichment culture techniques used in the isolation of the carboxydobacteria do not assess the population size. Even if such data were presented, this would not prove that the isolates were actively metabolizing CO in the soil environment. The carboxydobacteria are not obligate CO chemoautotrophs. These species can grow autotrophically with hydrogen as the electron donor or heterotrophically on a variety of substrates (17). Hence, their frequent occurrence in soils subjected to CO exposure could be the result of their growth at the expense of other pollutants at these sites.

Bartholomew and Alexander (2) hold the view that the nonspecific microorganisms are of importance in CO removal by soils. This conclusion was based largely on experiments in which the provision of CO did not increase the levels of $^{14}\text{CO}_2$ assimilation. An enhancement would have been expected if CO chemoautotrophs were involved.

This report will attempt to resolve the apparent contradiction by examining the adaptability of the soil sink to increasing levels of CO. After conducting a progression of experiments to ascertain the reliability of the assay methods for these complex systems, it was possible to examine the influence of CO exposure on the development of the active population(s). Finally, the question of specific versus nonspecific oxidation was addressed in studies which measured the assimilation of CO_2 and the dependence of this process on CO oxidation.

MATERIALS AND METHODS

Soil samples. With the exception of one sample collected at Sapelo Island, Ga., the soil samples were collected within an 80-km radius of Atlanta, Ga. The standard sampling procedure consisted of collecting a section of soil about 10 by 10 by 5 to 10 cm deep, clearing vegetation and plant litter from the surface, collecting the top 1 cm of the soil section, and forcing this soil through a brass sieve with 1-mm openings. The soils were then either stored under laboratory air (air-dried samples) or in a 5-liter desiccator kept at 100% relative humidity (RH).

Soil samples 205-A, 205-B, and 205-C were obtained in a different manner. After digging a pit down to a depth of about 10 cm, a brass cork borer (1.6 by 10

cm) was used to sample horizontal cores at depths of 0.8, 3.8, and 5.8 cm (measured from the surface to the center of the core). The samples were sieved and then stored under 100% RH.

Gas additions and incubations. Unless otherwise indicated, soil samples were removed from their storage vessel, and 0.3-g samples were weighed on stainless steel planchets (2.5 cm). The planchets with soil were placed in individual Pyrex incubation chambers, all having gas volumes of approximately 30 cm^3 . When humid incubation conditions were to be used, 0.5 ml of water was placed in a container inside the incubation chamber. Corning high-vacuum silicone grease was used to seal the chambers.

The vacuum system and details of the procedure for filling incubation chambers have been described elsewhere (H. G. Spratt, Jr., M.S. thesis, Georgia Institute of Technology, Atlanta, 1980). Briefly, the standard procedure involved evacuation of the incubation chamber and filling with air containing metered volumes of ^{14}CO and diluent ^{12}CO . The ^{14}CO was stored in a 600- cm^3 Pyrex reservoir which had to be loaded by expanding the contents of a break-seal ampoule into 380 torr of N_2 . The total amount of CO ($^{14}\text{C} + ^{12}\text{C}$) in the incubation atmosphere was between 55 and 58 nmol. The lower end of the range came after the reservoir had been sampled numerous times.

In the experiments with $^{14}\text{CO}_2$ the gas was generated by acidification of $\text{NaH}^{14}\text{CO}_3$, transferred to the metering cavity of the vacuum manifold, and then swept into the evacuated incubation chamber. The standard incubation atmosphere was air containing 60 nmol of $^{14}\text{CO}_2$ and ^{12}CO added as indicated. One experiment dealt with $^{14}\text{CO}_2$ incubations in a chamber where the only CO_2 present initially was that produced in the $\text{NaH}^{14}\text{CO}_3$ reaction. This was accomplished by mounting an Ascarite CO_2 scrubber between the reaction chamber and the outside air. The chamber was flushed with N_2 before the introduction of the incubation atmosphere.

Incubations were carried out at room temperature (approximately 23°C) for the times indicated in the various experiments. The incubations were terminated by sweeping the incubation gas from the headspace of the chambers as described below. The soil samples were removed from the chamber and stored at -20°C.

Gas collection and quantification. In all cases the gases quantified were ^{14}CO or $^{14}\text{CO}_2$ or both. For collection of CO_2 a stream of air (20 cm^3/min) was passed through the sample chamber for 15 min, sweeping the gas phase into a bubbler containing 1 ml of Hyamine hydroxide solution (1 M in methanol). The CO in the effluent of this trap was passed through a CuO column (0.35 cm [inside diameter] by 30 cm) heated to 650°C so as to combust CO to CO_2 . The CO_2 in the effluent from the CuO column was collected in a second bubbler containing 1 ml of Hyamine hydroxide. The Hyamine hydroxide was rinsed from the traps with 4 ml of methanol, and 0.2-ml samples were withdrawn and placed in scintillation vials along with 10 ml of scintillation cocktail (5 g of PPO [2,5-diphenylloxazole] in 1 liter of toluene). Samples were counted in a Beckman model LS 100C liquid scintillation counter for time periods needed to give a 2-sigma error

of less than 1%. From a knowledge of the counting efficiency (90.8%) and the specific radioactivity of the ^{14}CO , determinations of the amounts of ^{14}CO and $^{14}\text{CO}_2$ were made. In the $^{14}\text{CO}_2$ incorporation experiments, the incubation atmosphere was collected in one Hyamine hydroxide trap and quantified as above.

Periodic quantification of $^{14}\text{CO}_2$ contamination of the ^{14}CO reservoir was accomplished by the same gas collection procedure from a sample chamber containing no soil. We previously reported this spontaneous oxidation of ^{14}CO to $^{14}\text{CO}_2$ which occurs in Pyrex storage vessels (5). Over the 215 days that these studies were conducted, the level of contamination increased in a linear fashion from 1.7 to 7.8% of the total ^{14}C .

Quantification of $^{14}\text{CO}_2$ incorporation. The wet combustion method used was a modification of previously reported procedures (1, 16). Soil samples (0.3 g in all cases) were removed from the sample chamber, frozen, placed in a test tube (1.5 by 16 cm) and stoppered with a serum cap. The test tube was connected to a bubbler containing 2 ml of Hyamine hydroxide solution by means of a tube and 20-gauge hypodermic needle. A nitrogen gas line was connected to the tube through a second needle. The abiotically absorbed $^{14}\text{CO}_2$ in the soil was released by injecting 1.9 ml of a carbonate flush solution prepared by mixing 11.4 ml of concentrated H_2SO_4 and 18.4 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 120 ml of water. The tube was placed in a boiling water bath, and the nitrogen gas flow rate was adjusted to about 50 cm^3 per min. This inorganic $^{14}\text{CO}_2$ fraction was collected for 5 min. The nitrogen flow was stopped, and the tube was removed from the boiling water bath. The serum stopper was removed, 0.5 g of potassium dichromate was added to the tube, and the stopper was quickly replaced. A fresh Hyamine hydroxide bubbler was connected to the tube, and 1.5 ml of digestive acid (concentrated H_2SO_4 and 85% H_3PO_4 , 6:4) was added through the stopper with a syringe. The acid was added carefully to ensure that the reaction was not too vigorous. The nitrogen flow rate was again adjusted to about 50 cm^3 per min, and the tube was placed in the boiling water bath. Collection of the $^{14}\text{CO}_2$ resulting from combustion of ^{14}C -labeled organics present in the soil was for 5 min. The inorganic ^{14}C -fraction and the organic ^{14}C -fraction were each then rinsed from the bubblers with 4 ml of methanol, and 1.0 ml was withdrawn for determination of radioactivity as described above. The counting efficiency for these fractions was 88.4%. The extent of incorporation was calculated with corrections made for the dilution of the ^{14}C in ^{12}C gases as described briefly below and in more detail elsewhere (Spratt, M.S. thesis).

Gas chromatographic procedures. The gases separated by the combustion trapping system were confirmed to be CO and CO_2 by a gas chromatographic technique as described elsewhere (Spratt, M.S. thesis). Briefly, the method involved injecting excess CO_2 and CO into the incubation chamber to effectively stop the metabolism of the ^{14}C gases, separating CO_2 from the air and CO fraction on a silica gel column, combusting the effluent CO to CO_2 on a CuO column at 660°C and then separating this CO_2 (derived from CO combustion) from air on a second silica gel column. The two

CO_2 peaks were visualized with a thermal conductivity detector and collected in Hyamine hydroxide bubblers. The radioactivity of the two fractions was determined as described above.

CO enrichment procedure. Soil samples were removed from the regular storage vessel, and 4-g samples were weighed on plastic boats. The soils were then moistened by adding 0.4 ml of water, mixed thoroughly, and placed in a 2.5-liter desiccator along with a container of water to maintain 100% RH. The desiccator was sealed with silicone high-vacuum grease and evacuated to 15 torr. The desiccator was then connected to the vacuum manifold, whose metering cavity contained 24 torr of ^{12}CO . An outside port on the manifold was opened to allow the inrushing air to sweep the CO into the desiccator. The soils were exposed to the 15-torr vacuum for no longer than 5 min. This produced an atmosphere of air with 200 ppm (vol/vol) of CO in the desiccator. The CO enrichment incubations were for 21 days with the atmosphere changed four times by this same procedure. An enrichment control was performed by treating soils as above except that no CO was added. This enrichment control was incubated for 21 days with the evacuation procedure repeated four times.

Respiratory and background CO_2 determination. Incubations were set up using 0.3 g of soil samples in sample chambers at 100% RH. A serum stopper was placed in one of the 10/30 standard taper connectors on the chamber. At the indicated times, 0.5-cm^3 samples of the incubation atmosphere were withdrawn using a gas-tight syringe. The gas was injected into a Beckman 865 nondispersible IR gas analyzer through a section of tubing in a series with the nitrogen gas line leading to the sample window. For each analysis, five injections were made, and their results were averaged. Each chamber was sampled at only two time intervals so as to minimize the loss of incubation atmosphere through the punctured serum stopper. Calibration of the instrument was accomplished using 309 and 276 ppm CO_2 standards generously provided by E. Lloyd Dunn.

ATP extraction and analysis. Extraction of adenosine triphosphate (ATP) from soil samples was accomplished by a modification of the method of Paul and Johnson (13). The soil (0.3 g) was placed in a 50-ml Pyrex beaker to which was added 25 ml of 0.5 M NaHCO_3 (pH 8.5) and 3 ml of CHCl_3 . The beaker was positioned under a microprobe of a model W185C sonicator (Heat Systems Co.) lowered to about 2 mm above the bottom of the beaker. The mixture was sonicated at 95-W output for 30 s and then at 145 W for another 30 s. A conical centrifuge tube was then filled with the aqueous layer of the extract and centrifuged at about $2,000 \times g$ for 5 min. A 5-ml amount of the upper portion of the supernatant was transferred to a test tube (16 by 1.5 cm). This tube was plugged with a rubber stopper through which a section of glass tubing was inserted. This glass tube was connected to a vacuum source (300 torr) to facilitate the evaporation of the residual CHCl_3 . The tube was warmed in a water bath at 60°C for 1 to 5 min, i.e., until the bubbles forming on the inside of the tube were all the uniformly small CO_2 bubbles (from NaHCO_3). The larger bubbles

coming out before this were residual CHCl_3 . These extracts were stored at -20°C for periods not exceeding 5 days. When the ATP was to be determined, the samples were thawed and diluted with 25 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.8. The ATP content of these extracts was quantified on 30- μl samples of extract using the firefly assay as previously reported (6). A standard curve was prepared using 30- μl samples of ATP diluted in a Tris- NaHCO_3 solution in the proportions used for extraction. A plot of log light intensity versus log ATP concentration yielded a straight line over a range of 0.03 to 3 pmol of ATP.

Soil microorganism enumeration. Determination of viable microorganisms present in soil samples was made in growth tube experiments. The procedure was started with dilution of 0.1 g of soil in 10 ml of 0.01 M sterile potassium phosphate buffer (pH 7.0) in a test tube (16 by 1.5 cm). Two drops of Tween 80 surfactant were added, and the tube was mixed in a Vortex blender for 3 min. Serial dilutions were made with the phosphate buffer. Trypticase soy broth (TSB; BBL Microbiology Systems) was the growth medium. Replicates of five tubes of 10 ml of TSB were inoculated with 0.1-ml samples of the dilutions. The tubes were incubated at room temperature for 72 h and then scored either positive or negative. The numbers of viable organisms per gram of soil were determined by the standard most probable number technique of analysis (14).

Chemicals used. [^{14}C]sodium bicarbonate (specific radioactivity, 8.4 Ci/mol) was obtained from New England Nuclear Corp. [^{14}C]carbon monoxide (specific radioactivity, 56 Ci/mol) was from Amersham Corp. Scintanalyzer-quality PPO and the toluene for scintillation cocktail were from Fisher Scientific Co. ATP and Hyamine hydroxide (methyl benzethonium hydroxide) were from Sigma Chemical Co. The purified and stabilized luciferin-luciferase mixture was from Du Pont. All other chemicals used were of reagent grade and were obtained from the standard sources.

RESULTS

A series of experiments was conducted to ascertain the reliability of the assay methods. One line of evidence that the oxidation of CO to CO_2 is a biological process came from experiments comparing the activity of autoclaved and untreated soils (not shown). Soil samples autoclaved for 15 min were less than 1% as active as untreated ones.

A gas chromatographic technique was used to confirm that the ^{14}C -fractions detected by the standard combustion and alkaline trapping method were predominantly CO_2 and CO. After incubation of an active soil under the standard $^{14}\text{CO} + ^{12}\text{CO}$ atmosphere the $^{14}\text{CO}_2/^{14}\text{CO}$ ratios were determined by the combustion trapping method and by the gas chromatographic combustion trapping method. The ratios obtained by the two methods were essentially identical, i.e., 0.57 and 0.61. Thus, it seems unlikely that

gases other than CO_2 and CO represent significant portions of the ^{14}C -fractions detected by the standard combustion trapping method.

In several soils tested, the ambient moisture levels were inadequate to promote levels of CO oxidation which were representative of the total activity. Attempts to define a standard quantity of water needed for a specific quantity of soil were unsuccessful because of differences in the initial levels of water in the samples and in the amounts required for saturation. Another problem is presented due to the low solubility of CO in water. For example, only 41 pmol of CO is dissolved in 0.9 ml of water at 20°C under an atmosphere containing 40 ppm of CO. A comparison of the activities of humidified and moistened samples is given in Table 1. The humidified samples showed activities which increased approximately proportionally to the sample size. With the moistened soils, the amount of soil was varied, whereas the ratio of added water to soil was kept constant. These moistened samples gave lower activities, and the proportionality was not attained. For example, the 0.3-, 1.0-, and 3.0-g moistened samples were 0.6, 0.21, and 0.18 times as active as the comparably sized humidified samples. With the liquid water treatment, the overlying wet soil apparently interferes with CO diffusion. In contrast, the humidified condition apparently results in increased surface area of the water film, thereby promoting a more rapid diffusion of CO to the active species in the microenvironments of the soil particles. In other experiments, soil samples were equilibrated under various relative humidities in an attempt to determine the minimal moisture requirements for CO oxidation. With each of the four soils tested, the activity was not detected at an RH of

TABLE 1. CO oxidation by various amounts of moistened or humidified soil^a

Soil sample (g)	Water added	Oxidation of CO to CO_2	
		nmol/h	nmol/h per 0.3 g
0.3	100% RH	2.5	2.5
1.0	100% RH	12.6	3.78
3.0	100% RH	36.7	3.67
0.3	0.09 ml	1.5	1.5
1.0	0.3 ml	2.6	0.78
3.0	0.9 ml	6.5	0.65

^a The roadside soil (1127-C) was collected 1 to 2 m from Interstate 75/85 near downtown Atlanta, Ga., and then stored under 100% RH. The indicated amounts were placed on aluminum dishes 2.5 cm in diameter. Sample thicknesses were approximately 1, 2, and 6 mm for the 0.3-, 1.0-, and 3.0-g soil samples, respectively.

less than 90%, and maximal activity required an RH approaching 100%.

The above findings indicate that kinetic data are not easily interpreted in studies with moistened soils because the limited solubilization of CO in water will limit substrate availability. In all subsequent experiments, the measurements of CO oxidation were made with soils pre-equilibrated and incubated at 100% RH. Other experiments with humidified samples of roadside soil 1011-B indicated that the CO oxidation reaction exhibited saturable kinetics (data not given). A Lineweaver-Burk plot (10) showed the substrate concentration which promoted half-maximal velocity (K_m) to be 14.9 ppm of CO in the headspace. The maximal velocity (V_{max}) was determined to be 14.1 nmol/1.5 h per 0.3 g of soil. Since the level of CO used in the standard assay is 40 to 41 ppm (approximately 58 nmol), the substrate should not be limiting in the course of a 3-h experiment. The highest rate of CO oxidation observed using a freshly collected, roadside soil (313-A) was 32.5 nmol/1.5 h (Table 2). With soils in this range of activity, substrate limitation should not be a factor in experiments of 2-h duration.

Figure 1 shows a time course study with samples of soil 313-A which had been stored 25 days at 100% RH. The linear response with time indicates that such stored samples can be used in kinetic studies. However, the rate of CO oxidation in Fig. 1 was 70% lower than that observed with a freshly collected sample (Table 2). This loss of activity during storage was also observed with other soil samples, being particularly pronounced in samples from roadside sites (data not shown). In the case of roadside samples, this storage removed the soils from an

environment of higher levels of atmospheric CO. If species were originally present which could utilize CO as a carbon or energy source or both, the survival of the population would be dependent on the ability of the species to make use of alternative carbon sources. Otherwise, the population would decline as a consequence of selective pressures. A decline in the capacity of the soils to oxidize CO during storage would also be expected if CO serves as an inducer of the oxidative enzyme even if the cell derives no benefit from the reaction. A decline in CO oxidation activity would not be expected if the reaction is an example of cometabolism (4); i.e., the enzyme is specific for or is produced in response to other substrates and its oxidation of CO is coincidental.

A systematic investigation of the decline in activity during storage and its restoration by the intentional introduction of CO is shown in Fig. 2. Carbon monoxide oxidation by this roadside soil was followed for a period of nearly 2 months' storage in the laboratory under 100% RH. Within 1 week after collection, a 30% decline in activity was seen. After approximately 5 weeks of storage, the CO oxidation was only 21% of its initial value. The soil was under an atmosphere which was allowed to equilibrate with laboratory air every 3 days. However, this storage did remove the soil from the elevated CO levels at the roadside site. An enrichment study was performed on the 14th day after collection by transferring a sample of the soil to another storage chamber containing 200 ppm of ^{12}CO . This atmosphere was replenished periodically. After 3

TABLE 2. CO oxidation by soils collected at various sites

Soil	Collection site	Oxidation of CO to CO ₂ (nmol/1.5 h)
1127-A	Floodplain, Clarkston, Ga.	0.75
1212	Uncultivated grassland, Carrol Co., Ga.	4.5
1217	Salt marsh, short spartina, Sapelo Is., Ga.	0.35
1127-C ^a (roadside)	Near Interstate 75/85 ^b	9.0
102-A (roadside)	Near surface street ^b	16.1
313-A (roadside)	Near Interstate 75/85 ^b	32.5

^a CO oxidation was measured after 12 days of storage at 100% RH. All others were analyzed within 1 day after collection.

^b Collected from sites within 1 to 2 m from roads within a 2-km radius of downtown Atlanta.

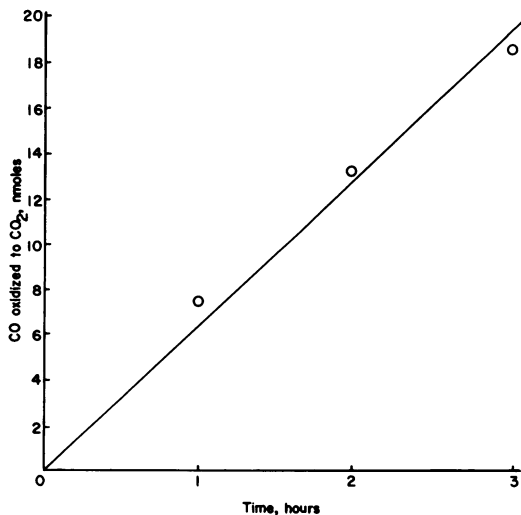


FIG. 1. Oxidation of CO with time. Soil 313-A (see Table 2) was tested after 25 days' storage under 100% RH.

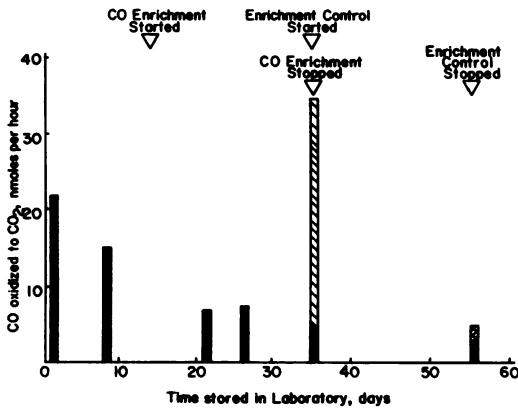


FIG. 2. CO oxidation by soil 313-A after storage with or without a CO-enriched atmosphere. Soil 313-A (see Table 3) was stored under 100% RH with the container opened and allowed to equilibrate with fresh laboratory air every 3 days. The enrichments were conducted using samples from this stored sample. Symbols: ■, stored sample, not enriched; ▨, stored sample, enriched under 200 ppm of CO; ▩, stored sample, enrichment control (no CO).

weeks of enrichment incubation, the soil was removed, and its activity was measured. After 35 days' total storage, the enriched soil showed CO oxidation rates more than seven times greater than the same soil stored under laboratory air. Even more impressive is the fact that this enriched soil oxidized CO more rapidly than a freshly collected sample.

The methods for the standard storage and the CO enrichment were different in one respect. In the latter condition, the procedure for changing the atmosphere involved evacuating the vessel to a pressure of about 15 torr before refilling with air containing 200 ppm of CO. To ascertain that this evacuation treatment was not responsible for the enhancement in CO oxidation, an experiment was set up on the 35th day after collection using an evacuation procedure but refilling with laboratory air (without added CO). The results of this control experiment for the enrichment study are given in Fig. 2. The sample of soil 313-A which had been subjected to evacuation to 15 torr four times over a 3-week period did show 83% more activity than the stored sample. The difference is hardly significant when compared with the sample incubated under 200 ppm of CO. Also, it should be noted that there was not a net increase in CO oxidation capacity during the period of enrichment control incubation. The reason for the higher activity in the enrichment control than in the stored sample is not known.

Results of the enrichment studies suggested

the possibility of a higher incidence of CO-oxidizing species in soils from locations with higher ambient CO levels. Thus, differences in capacity for CO oxidation should be observed between CO-polluted (roadside) soils and relatively unpolluted (field) soils. Samples taken outside Atlanta's perimeter highway (I-285) in fields away from roads (1127-A and 1212) and a sample from Sapelo Island, Ga., were compared with soil samples taken from roadside sites in Atlanta (Table 2). There was some variation in activities among samples within the two broad categories. However, the minimum of a 2-fold difference and the maximum of a 93-fold difference indicate a much greater CO-oxidizing activity in the Atlanta roadside soils.

Obviously, these differences between different soils are not necessarily the result of exposure to CO. Table 3 shows another comparison, i.e., looking for spatial distribution of the active organisms which reflects lower CO availability with depth. With this roadside soil, the highest rate of CO oxidation was seen in the horizontal soil core collected nearest the surface. The decrease in activity with depth was not accompanied by a decrease in the population of microorganisms culturable under aerobic conditions. Apparently, at the 5-cm depth the limited diffusion of CO is insufficient to promote the enrichment of the CO oxidizers. Oxygen limitation should not be as severe. The greater abundance of O₂ (ca. 20%) than CO (ca. 1 ppm) should promote the solubilization of considerably greater amounts of O₂ in the aqueous phase of the subsurface soil.

Differences in the rates of CO oxidation between roadside soils and field soils and in enriched soils compared with their enrichment controls suggest that the responsible organisms do respond to a history of exposure to elevated levels of CO. The obvious question is whether this exposure promotes a greater total population or a selective proliferation of the species which oxidize CO. Thus, experiments were de-

TABLE 3. CO oxidation by soils collected at various depths

Soil ^a	Depth of collection site (cm)	Organisms present ^b ($n \times 10^6$)	Oxidation of CO to CO ₂ (nmol/1.5 h)
205-A	0.8	7.0	5.3
205-B	3.8	4.9	1.8
205-C	5.8	4.6	0.7

^a Collected at the indicated depths from a pit dug 2 m from Interstate 75/85 near downtown Atlanta.

^b Multiple-tube fermentation using most probable number analysis.

signed to examine the relationship of the biomass of the soils and their capacity to oxidize CO. One indicator used was the numbers of viable organisms as determined in tube dilutions and most probable number analysis (data not shown). The basis for comparison was the specific activity, i.e., nanomoles of CO oxidized per hour per million culturable organisms. The specific activities of roadside soils 1011-B, 1127-C, and 102A were 43.6, 60.3, and 45.3, respectively, whereas the values for field soils 1127-A and 1212 were 0.5 and 3.0, respectively. One interpretation of these differences is that the CO-oxidizing species in the roadside soils comprise a greater fraction of the microbial population than do those in the field soils. Obviously, this interpretation is subject to criticism on the grounds that the enumeration technique may not yield a valid estimate of the total microbial population in all types of soils. For example, such differences in specific activity could be seen if the culturable fraction in the roadside soils is smaller than the culturable fraction in field soils.

An alternative method chosen was the estimation of biomass by determining the ATP content in extracts of the soil. The ATP analysis on extracts of field soil 1127-A and roadside soil 1127-C is given in Table 4. For the four analyses on these soils, the values fell in a narrow range of from 0.73 to 1.15 μg of ATP per g of soil. Thus, the large differences in the capacity of the soils to oxidize CO cannot be explained by the small differences in the total biomass of the soils. In the enrichment experiments there was not an appreciable change in the ATP levels detected in soils 1127-A (+12%) or 1127-C (-3%) in spite of the dramatic increases in the capacity for CO oxidation. This is illustrated in the specific activity values based on the ATP content. As a result of the enrichment treatment, the specific activities were increased by about eightfold and fivefold for soils 1127-A and 1127-C, respectively. If the increase in CO oxidative capacity upon

prolonged exposure to CO is the result of an increase in the population of the responsible organisms, the species must not comprise a major portion of the total microbial community in these soils.

The inference that the responsible organisms derive benefit in the course of the oxidation of CO to CO_2 was noted in previous discussions. One such possibility is that energy is conserved during the oxidation, and this energy drives the assimilation of CO_2 , i.e., a chemoautotrophic (chemolithotrophic) process. Since the CO-oxidizing species apparently comprise a minor proportion of the total microbial population, it was impractical to attempt to quantify increases in ATP content which accompany the oxidative reaction. An increase in the adenylate charge in these few cells would not cause a measurable increase in the ATP content of the total ecosystem. A measurement of the assimilation of ^{14}C into organic constituents proved to be more practical. However, this too is complicated by the fact that assimilation of $^{14}\text{CO}_2$ by heterotrophs and other types of autotrophs (CO-independent) provides a background level for which corrections must be made.

Experiments were designed to measure any enhancement of CO_2 assimilation when CO oxidation was occurring. There is a problem in determining exactly how much assimilation is occurring because of the dilution of the $^{14}\text{CO}_2$ in $^{12}\text{CO}_2$ contributed by cellular respiration in the soil and the $^{12}\text{CO}_2$ present in air. The importance of an accurate determination of this dilution of the $^{14}\text{CO}_2$ in $^{12}\text{CO}_2$ is obvious. Moreover, the $^{12}\text{CO}_2$ contributed by respiration is changing with time (Fig. 3). In the cases where CO was included in the headspace of the test chambers, the oxidative reaction contributed an additional increment of CO_2 which will also change with time. Wet combustion analyses indicated that in soil 313-A there was not a measurable fraction of the CO_2 which had undergone exchange into soil carbonates or had been absorbed by soil particles. In our modification of the combustion method, the abiotically exchanged or absorbed CO_2 liberated during the carbonate flush is collected, and its radioactivity is determined. For samples of soil 313-A incubated under the varied conditions in Fig. 4, this liberated ^{14}C was an almost constant 12% of the ^{14}C released during the combustion step. This percentage can be attributed to the acid destruction of cellular organic constituents during carbonate flush. About 10% of the assimilated ^{14}C is released as $^{14}\text{CO}_2$ during the carbonate flush treatment (Hubbard, unpublished data). Thus, there was no added complication of correcting for dilutions

TABLE 4. Biomass measurement and CO oxidation

Soil ^a	CO^b enrichment	ATP ($\mu\text{g}/\text{g}$ of soil)	Sp act (nmol of CO oxidized to $\text{CO}_2/\mu\text{g}$ of ATP per h)
1127-A	No	0.73	2.95
1127-A	Yes	0.82	23.9
1127-C	No	1.15	12.7
1127-C	Yes	1.12	67.7

^a Soils were collected (see Table 2), sieved, and then stored at 100% RH for 91 days.

^b When so indicated, the soils were placed under an atmosphere containing 200 ppm of CO and 100% RH for the last 21 days of the storage period.

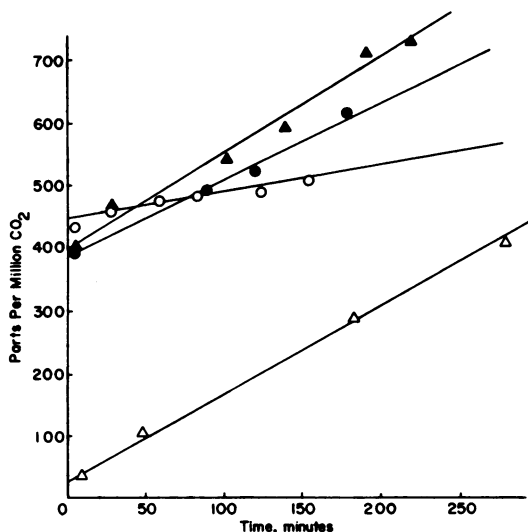


FIG. 3. Examples of measurements of respiratory CO_2 production by soil 313-A. Determinations were made on 0.3-g samples of soil incubated under 100% RH. Symbols: ●, soil after 11 days' storage; ○, soil after 21 days of CO enrichment (35 days' storage); △, soil under CO_2 -free air (after 15 days' storage); ▲, soil under 40 ppm of ^{12}CO (after 8 days' storage).

caused by exchange of gaseous CO_2 into the soil carbonates.

In the five sets of experiments summarized in Fig. 4, the CO-independent, CO_2 assimilation is indicated by the incorporation into cell material when $^{14}\text{CO}_2$ alone was added. Supplementation of the $^{14}\text{CO}_2$ -containing atmosphere with ^{12}CO stimulated the assimilation of CO_2 into cell material by 47, 24, and 23% for samples tested after 1, 5, and 18 days' storage, respectively. The 18-day sample was run under different conditions in that the atmospheric CO_2 was removed from the air before the incubation was initiated. This was done to minimize any errors that might have been made in correcting for the dilution of $^{14}\text{CO}_2$ in atmospheric $^{12}\text{CO}_2$.

An even higher level of assimilation was noted when the incubation atmosphere contained ^{14}CO and ^{12}CO (Fig. 4). Here the unlabeled CO_2 substrate was provided from three sources, atmospheric CO_2 , respiratory CO_2 , and the oxidation of CO to CO_2 . The $^{14}\text{CO}_2$ was provided from the $^{14}\text{CO}_2$ contamination in the ^{14}CO gas supply and from the oxidation of ^{14}CO . The level of $^{14}\text{CO}_2$ contamination in the ^{14}CO reservoir was between 7.0 and 7.8% during the times that these experiments were performed. Since the oxidative reaction which converts CO to CO_2 is linear over these time intervals (see Fig. 1), the amount of $^{14}\text{CO}_2$ contributed from this source can be cal-

culated. However, the dilution calculations assume that the $^{14}\text{CO}_2$ is uniformly distributed throughout the incubation chamber. It is conceivable that the $^{14}\text{CO}_2$ derived from ^{14}CO oxidation is localized and is assimilated before being fully diluted in the total CO_2 pool. If so, the calculations of the level of CO_2 assimilation in the $^{14}\text{CO} + ^{12}\text{CO}$ condition may be a slight overestimation. By the same reasoning the calculation of CO_2 assimilation in the $^{14}\text{CO}_2 + ^{12}\text{CO}$ condition might be a slight underestimation because of the enrichment of $^{12}\text{CO}_2$ over $^{14}\text{CO}_2$ in the microenvironments of the soil. Nevertheless, these data present a strong argument for a CO-dependent assimilation of CO_2 .

Figure 4 also shows a series of $^{14}\text{CO}_2$ assimilation experiments run on a sample of soil 313-A which had been subjected to the laboratory enrichment treatment. Here again the CO_2 assimilation process was stimulated by including CO in the incubation atmosphere, and the highest activity was seen in the $^{14}\text{CO} + ^{12}\text{CO}$ condition. The reason for the relatively low-level (21%)

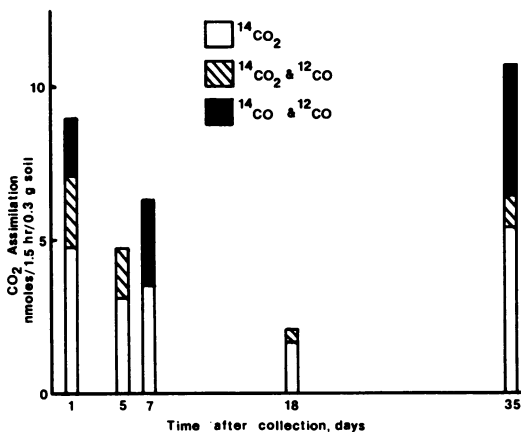


FIG. 4. CO stimulation of the incorporation of CO_2 into cell material. All tests were run with soil 313-A after the indicated storage times under 100% RH. The levels of $^{14}\text{CO}_2$ assimilation were determined by the modified wet combustion method. The total CO_2 ($^{12}\text{C} + ^{14}\text{C}$) was calculated by correcting for the dilution of the available $^{14}\text{CO}_2$ (that added and that produced from ^{14}CO oxidation) in the $^{12}\text{CO}_2$ from the atmosphere, from endogenous respiration of soil microorganisms and from the oxidation of ^{12}CO by soil microorganisms. For the 18-day samples the CO_2 was removed from the atmosphere immediately before the incubation was initiated by adding $^{14}\text{CO}_2$ or ^{14}CO and ^{12}CO . The 35-day samples had been enriched under 200 ppm of CO for the last 21 days of the storage period. The $^{14}\text{CO} + ^{12}\text{CO}$ condition was not tested on the 5- and 18-day stored samples. The $^{14}\text{CO}_2 + ^{12}\text{CO}$ condition was not tested on the 7-day stored sample.

enhancement of CO₂ assimilation by ¹²C can be explained in part by the fact that all the available CO had been oxidized to CO₂ during the 2-h incubation; i.e., the driving force had been depleted. This experiment provides additional evidence that a CO-enriched atmosphere is selective for species which benefit from the oxidative reaction.

DISCUSSION

The question of possible benefit that the responsible microorganisms may receive from CO oxidation was addressed in two ways. If there are organisms present which conserve energy during CO oxidation and drive the assimilation of CO₂ with this energy (chemoautotrophs), as has been proposed by Zavarzin and Nozhevnikova (17), these organisms should be found in greater numbers wherever concentrations of CO are high. Thus, the simplest way of looking for an enhancement was the comparison of soils collected from areas of diverse ambient CO levels.

The rates of CO oxidation seen with roadside soils were consistently higher than those with field soils from outlying areas. One interpretation of this finding is that organisms which oxidize CO profit from the higher levels of the pollutant. The ability to grow autotrophically would selectively enrich such species over other microbial populations in the soil. However, consideration should be given to the fact that the exhaust from internal combustion engines contains not only CO but also other incompletely burned hydrocarbons. Conceivably, the organisms which grow on the hydrocarbon substrates could be capable of oxidizing CO coincidentally. The observation of marked increases in the capacity of soils to oxidize CO after the laboratory enrichment treatment precludes a relationship between CO oxidation and other gaseous pollutants.

The second line of investigation into possible benefit to the responsible microorganisms was the comparison of the incorporation of ¹⁴C into cellular constituents under various ¹⁴CO, ¹⁴CO₂, ¹²CO, and ¹²CO₂ atmospheres. The observation of enhanced CO₂ incorporation in five different experiments adds credibility to the hypothesis that the energy conserved during CO oxidation was being used to drive CO₂ assimilation.

The above observations are in contrast to the findings of Bartholomew and Alexander (2). In studies with Williamson silt loam soil, they did not detect an enhancement of CO₂ assimilation caused by the presence of CO and thus concluded that autotrophs were not of importance in the removal of CO. One possible explanation

for the different conclusions is that the Williamson silt loam is not particularly active in terms of CO oxidation. It is not clear whether a small amount of CO-dependent assimilation of CO₂ could have been distinguished from the CO-independent processes. Moreover, in their results no mention was made of ¹²CO₂ released from the soil into the incubation atmosphere by microbial respiration. Compared with the results presented here showing a production of more than 200 ppm of ¹²CO₂ (268 nmol) in 3 h for only 0.3 g of soil, their incubations employed 5 g of soil for 24 h. Even though initially under CO₂-free air, the atmosphere could have had an appreciable amount of ¹²CO₂ within a few minutes. In all of the CO₂ assimilation experiments presented herein, correction was made for respired CO₂, background atmospheric CO₂ (if applicable), and that produced from CO oxidation.

Another explanation for the difference between our results and those of Bartholomew and Alexander (2) may be in the histories of exposure of the soils to CO. Our evidence for the autotrophic process came in tests with a highly active roadside sample. It remains to be determined whether substantial populations of the autotrophs can develop in soils exposed to "clean air" levels of CO.

An attempt was made to compare the proposed stoichiometry for utilization of CO by carboxydobacteria (11, 12) with the results presented herein. When tested 1 day after collection, soil 313-A oxidized 32.6 nmol of CO to CO₂ per 1.5 h and incorporated 2.3 nmol of CO₂ per 1.5 h via a method other than the CO-independent assimilation, i.e., the difference between the CO₂ incorporation under ¹⁴CO₂ + ¹²CO and that under ¹⁴CO₂ alone. This gives a ratio of assimilation to oxidation of 1:14. A ratio of 1:34 was observed in tests run with this soil at 5 days after collection, and a ratio of 1:37 was observed in tests with the enriched sample. From studies with growing cultures of carboxydobacteria, Nozhevnikova and Yurganov (12) reported a ratio of 1:24, whereas Meyer (11) reported a higher ratio of 1:6. Our findings are more in line with the lower value but, as noted above, these data may be slightly in error due to the nature of the calculations used in correcting for dilutions. Moreover, there is no a priori reason to assume that all carboxydobacteria follow the same stoichiometry during all phases of metabolism and growth.

The apparent link between greater CO₂ incorporation and CO oxidation, the enhancements seen in CO oxidation by city soils over field soils, and the marked increase in CO oxidation after enrichment incubations all favor the hypothesis

that the responsible organisms are autotrophs. An even more convincing argument could be made with an *in situ* demonstration that the carboxydobacteria are actively metabolizing CO in these soils.

In the only sample examined kinetically, roadside soil 1011-B, the oxidative reaction exhibited Michaelis-Menten kinetics with a K_m value of 14.9 ppm of CO. Data from the Georgia Department of Natural Resources for four sampling stations within a 4-km radius from this site showed the mean CO level for 1979 to range from 1.4 to 1.8 ppm. The highest 1-h average values for these stations ranged from 17 to 23 ppm. Without other considerations the CO-oxidizing organisms would appear to be provided with adequate CO levels during the peak periods of pollution and not be severely substrate limited at other times. However, before extrapolating from these findings, consideration should be given to the effects of moisture levels in the soil on the availability of CO. The highest rates of CO oxidation were observed in soils equilibrated and incubated at 100% RH. The presence of liquid water apparently limits the diffusion of CO through the soil. This effect was detectable in soil samples 1 to 2 mm thick and was more pronounced in samples of greater thickness. Needless to say, there are probably few natural situations where the optimum conditions prevail. *In situ* measurements under the normal range of ambient conditions will provide the only meaningful estimates of the capacity of soils to remove CO from polluted atmospheres.

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