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# Role of Nanoporosity and Hydrophobicity in Sequestration and Bioavailability: Tests with Model Solids

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Phenanthrene was rapidly and extensively mineralized by a bacterium in the presence of glass or polystyrene beads with no pores, silica beads with 2.5−15 nm pores, 3-aminopropyl-bonded silica beads with 6-nm pores, and diatomite beads with 5.4 $\mu$m pores. These beads sorbed 10−99% of the compound in 15 h, but 48−100% of the sorbed hydrocarbon was desorbed in 240 h. Although little phenanthrene was desorbed from octadecyl-bonded silica beads with 6-nm pores, the hydrocarbon was rapidly and extensively degraded. In contrast, the bacterium mineralized <7% of the phenanthrene sorbed to polystyrene beads with 5- or 300−400-nm pores, and little of the compound was desorbed. These findings are consistent with the hypothesis that sequestration and reduced bioavailability occur when hydrophobic compounds enter into nanopores having hydrophobic surfaces.

## Introduction

The toxicities of a pollutant in soil and solution are often different because of the frequently lower bioavailability of the compound when present in soil. One of the mechanisms of reduced bioavailability is sequestration or aging. Organic compounds that persist in soil may become, with time, less available for biodegradation (*1*, *2*), less toxic to insects (*3*) and plants (*4*), less readily assimilated by earthworms (*5*), and less readily removed by mild extractants (*6*, *7*). Nevertheless, the compounds are still present, as indicated by their removal from soil by vigorous extraction with organic solvents (*2*, *5*). These data suggest that the compounds are becoming sequestered in the soil matrix. Nevertheless, the mechanism or mechanisms of sequestration are uncertain.

One mechanism that has been proposed is the entry of the organic compound into nanopores (*1*, *8*). Pores with diameters less than 100 nm have been shown to exist in a variety of dissimilar soils (*9*, *10*). A molecule that is deposited and entrapped in a nanopore with a diameter smaller than 100 nm probably is unavailable to any living organism since even the smallest bacterium has larger dimensions. However, the organic compound might diffuse readily out of the nanopore to a site where it is freely available to an organism. On the other hand, if the compound were sorbed to the surfaces of the nanopore, its diffusion to a microsite accessible to the organism might be retarded to such an extent as to account for sequestration.

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In environments as physically and chemically complex as soils, it is difficult to clearly define the mechanism of a process or processes that account for a time-dependent diminution in bioavailability, particularly since physical, chemical, as well as biological factors are all simultaneously involved. However, studies with model systems may provide insights into the responsible mechanism. In a previous investigation, models were used to explore the possible role of partitioning of organic molecules into solid humic materials as a possible cause of sequestration, and preliminary data were obtained that suggest a role of entrapment within nanopores (*11*). The present investigation was designed to determine the possible importance of hydrophobicity of nanopores in affecting the bioavailability of hydrophobic compounds. For this purpose, phenanthrene was selected as the test compound, and bioavailability was assessed in terms of the availability of the hydrocarbon to a microorganism. The IUPAC classification categorizes pores with diameters >50 nm, 2−50 nm, and <2 nm as macropores, mesopores, and micropores, respectively (*12*). Because this classification is inconsistent with usage in soil science, where macropores, mesopores, and micropores are often classified as having diameters of >75, 30−75, and 5−30 $\mu$m, respectively (*13*), the term nanopore is here used to indicate diameters smaller than those commonly considered by soil scientists as micropores.

## Experimental Section

Unlabeled phenanthrene and [9-$^{14}$C]phenanthrene (8.3 or 13.1 mCi/mmol, 98% purity) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were reagent grade.

Nonporous glass beads (44−66 $\mu$m diameter; Cataphote Corp., Jackson, MI) were suspended in 0.1 N HCl and autoclaved for 2 h to remove surface alkali. The beads were then washed with distilled water until the pH of the suspension was approximately 6.5. Porous silica beads and 3-aminopropyl-bonded (C3-) and octadecyl-bonded (C18-) silica beads were obtained from Supelco, Inc. (Bellefonte, PA); the surface areas were 550 and 300 $m^2/g$ for 6- and 15-nm pore-sized beads, respectively. Porous polystyrene beads (Chromosorb 103 and 106 with surface areas of 15−25 and 700−800 $m^2/g$, respectively) and calcined diatomite beads (Chromosorb P or dimethyldichlorosilane-bonded Chromosorb P; 4.0 $m^2/g$ surface area) were purchased from Sigma Chemical Co. Because of the basic surface of Chromosorb 103, the beads were acid-treated as described above. For nonporous polystyrene beads, polystyrene flakes (Scientific Polymer Products, Ontario, NY) were ground with a mortar and pestle and sieved to obtain particle sizes with diameters of 170−250 $\mu$m. The beads and sorbents were autoclaved for 2 h and dried at 100 °C for at least 24 h. The properties of the particles were provided by the suppliers.

Isolate P5-2 is a short, Gram-negative rod that can multiply using sorbed phenanthrene as its sole C source. This bacterium was originally isolated because of its ability to use phenanthrene sorbed to polyacrylic beads containing 9-nm pores (*14*). It was grown at 30 °C on a rotary shaker (120 rpm) in a 250-mL flask containing 100 mL of a medium composed of 1.0 g of phenanthrene, 0.10 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeCl_3$, 0.10 g of $MgSO_4 \cdot 7H_2O$, 0.10 g of $NH_4NO_3$, 0.20 g of $KH_2PO_4$, and 0.80 g of $K_2HPO_4$/L of distilled water. After 3 days, the culture was passed through a Whatman No. 3 filter to remove remaining phenanthrene crystals, and the filtrate was centrifuged at 7650*g* for 10 min. The cells were

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**TABLE 1. Sorption and Desorption of Phenanthrene[a]**

| bead type | bead diam ($\mu$m) | pore diam (nm) | phenanthrene sorbed (%) | | | phenanthrene desorbed (%) | |
|---|---|---|---|---|---|---|---|
| | | | 1 h | 5 h | 15 h | 5 h | 240 h |
| glass | 44−66 | none | 27.2A | 27.2A | 12.5A | 70.6A | 96.8A |
| silica | 70−140 | 2.5 | 45.3AB | 49.7BC | 57.2D | 60.5ABC | 96.4A |
| | 70−140 | 6 | 43.3ABC | 49.7BC | 44.0C | 66.8ABC | 100.8A |
| | 70−140 | 15 | 44.7AB | 46.2B | 50.1CD | 62.3ABC | 92.2AB |
| diatomite | 170−250 | 5400 | 54.9C | 58.0C | 48.3CD | 54.8C | 92.2AB |
| C2-diatomite | 170−250 | 5400 | 83.8D | 87.1D | 77.4E | 18.9D | 69.2B |
| C3-silica | 40−63 | 6 | 40.2AB | 35.0A | 37.4B | 59.6B | 102.5A |
| C18-silica | 40−63 | 6 | 94.2D | 97.3EF | 99.4G | 0.9E | 5.8D |
| polystyrene | 170−250 | none | 90.7D | 92.3D | 94.0F | 16.5D | 48.0C |
| | 170−250 | 5 | 98.0F | 99.4F | 99.7G | 0.4E | 3.0D |
| | 170−250 | 300−400 | 96.8E | 94.7E | 97.8F | 0.9E | 3.3D |

[a] Values are the means of duplicate determinations. The values in each column followed by the same letter are not significiantly different ($P < 0.05$).

washed twice with the inorganic salts solution adjusted to pH 5.7, and an inoculum of approximately $10^8$ cells was used to test mineralization.

To measure sorption, a solution of 2 $\mu$g of unlabeled and 1 $\mu$g of radioactive phenanthrene (ca. $1.0 \times 10^5$ dpm) in methylene chloride was added to a 15-mL Teflon-lined glass vial. The solvent was allowed to evaporate, and 3.0 mL of inorganic salts solution was added. At this concentration, the phenanthrene was all water soluble. The solution was mixed for 1 min at high speed with a vortex mixer, 0.3 g of sterilized beads was added, and the vials were shaken with a wrist action shaker for 1, 5, or 15 h at 21 ± 1 °C. After removal of the beads by centrifugation at 1100*g* for 15 min, 1.0 mL of the water phase was removed, and the concentration of phenanthrene in the aqueous phase was determined by measurement of radioactivity using a liquid scintillation counter (Model LS 7500; Beckman Instruments, Inc., Irvine, CA). By this procedure, 97.4% of the phenanthrene added to vials without sorbents was recovered in the aqueous phase after 15 h of shaking.

To measure desorption, phenanthrene (3.0 $\mu$g containing ca. $1.0 \times 10^5$ dpm of $^{14}C$ in dichloromethane) was added to 0.3 g of beads in a 7-mL glass vial. The solvent was allowed to evaporate, and after 1 h, a 0.01 M $CaCl_2$ solution containing 0.02% $HgCl_2$ was added to the top of the vials. The $HgCl_2$ was added to maintain sterility for the long period during which desorption was determined. For nonporous polystyrene beads, which would have coalesced in the presence of dichloromethane, the phenanthrene was dissolved in 6.5 mL of 0.01 M $CaCl_2$ solution containing 0.02% HCl, and then the beads were introduced. The glass vials were closed with Teflon-lined caps and shaken at 100 rpm at 21 ± 1 °C for up to 240 h. At regular intervals, 6.0 mL of the solution was removed, and the amount of desorbed phenanthrene was determined. At the same time, 6.0 mL of $HgCl_2$-containing solution of $CaCl_2$ was added to the vial. The cumulative values for desorption are presented below.

For tests of mineralization, vials containing 0.3 $\mu$g of phenanthrene and 3 mL of inorganic salts solution were prepared as described above. To reduce the solubility of the $CO_2$ formed by mineralization, the medium was supplemented to give a final concentration of 0.90 g of $KH_2PO_4$ and 0.10 g of $K_2HPO_4$/L to give a pH of 5.7. Beads (0.3 g) were added to the vials and incubated with phenanthrene for 1 h on a rotary shaker (100 rpm) at 21 ± 1 °C to permit sorption to occur. In the 1-h period, the extent of sorption had usually approached the maximum value. Bacterium P5-2 (approximately $10^8$ cells) then was added to the vials; this density was sufficiently high that the cell number would not increase appreciably given the low phenanthrene concentration. Each vial was sealed with a Teflon-lined silicone stopper through which was placed an 18-gauge hypodermic needle and a 16-gauge steel cannula. From the cannula was suspended a small vial containing 1.5 mL of 0.5 N NaOH to trap $^{14}CO_2$ released in mineralization. The vials were incubated at 30 °C on a rotary shaker operating at 120 rpm, and $^{14}CO_2$ formation was determined for 120 or 150 h by periodically removing the NaOH and replacing it with fresh solution. The NaOH removed was mixed with 4.5 mL of Liquiscint scintillation cocktail (National Diagnostics, Inc., Atlanta, GA), and the radioactivity was measured by liquid scintillation counting. The cumulative values for $^{14}CO_2$ evolution are presented below.

The pH of the bead−liquid mixture before mineralization ranged from 5.6 to 6.9, except that the pH in mixtures containing C3-silica beads was initially 8.8. The pH of mixtures with the C3 beads was adjusted to 6.5 with $H_3PO_4$. After 120 or 150 h of mineralization, the pH values of the bead suspensions ranged from 5.2 to 7.8.

## Results

**Sorption and Desorption.** To determine the sorption of phenanthrene, the percentage of phenanthrene left in the aqueous phase was measured at 1, 5, and 15 h, and the concentrations in the aqueous phase were used to calculate the amount of the compound that was sorbed. Within 1 h, the values for percentage sorbed usually reached maximum values (Table 1). Less phenanthrene was sorbed by glass and silica beads than by C18-silica and polystyrene beads. The surfaces of the glass and silica beads are hydrophilic, whereas the surfaces of the latter two bead types are hydrophobic. The amount of phenanthrene sorbed after 15 h was not significantly different ($P < 0.05$) from the amounts sorbed at 1 and 5 h, except that the extents of sorption after 15 h by C18-silica beads and the two types of porous polystyrene were significantly greater than at 1 h and the extents by glass and C2-diatomite were significantly smaller. The reason for the anomalous decline in the amount sorbed by the glass beads at 15 h is unknown. Even the nonporous polystyrene beads, which have hydrophobic surfaces, sorbed much of the test compound. Moreover, although the silica beads have pores of 2.5−15 nm and surface areas >300 $m^2$/g, they have a relatively low sorption capacity. On the other hand, C18-silica beads and porous polystyrene beads had sorbed more than 97% of the phenanthrene at 15 h. The extent of sorption was highest with the porous hydrophobic beads made of polystyrene, but even the diatomite particles with 5.4-$\mu$m pores and a surface area of only 4.0 $m^2$/g sorbed approximately half of the compound.

Measurements were also made of the amount of phenanthrene desorbed at 5 and 240 h. The particles were not those used in tests of sorption. The solutions used for the analyses

**TABLE 2. Effects of Beads on Maximum Rates and Extents of Phenanthrene Mineralization by Bacterium P5-2[a]**

| bead type | pore diam (nm) | rate (%/day) | extent (%) |
|---|---|---|---|
| none | NA[b] | 59.2A | 67.9A |
| glass | none | 59.6A | 64.7AB |
| silica | 2.5 | 56.7AB | 65.5ABC |
| | 6 | 53.6AB | 64.8ABC |
| | 15 | 45.4ABC | 60.5BC |
| diatomite | 5400 | 43.5C | 59.9C |
| C2-diatomite | 5400 | 42.0C | 59.2C |
| C3-silica | 6 | 51.6B | 63.7ABC |
| C18-silica | 6 | 44.6BC | 58.4BC |
| polystyrene | none | 44.4C | 57.4ABC |
| | 5 | 0.9D | 2.5D |
| | 300−400 | 2.0D | 6.6E |

[a] Values are the means of duplicate determinations. The values in a column followed by the same letter are not significantly different ($P < 0.05$). [b] Not applicable.

were removed and replaced periodically. In the first 5 h, more than half of the compound had desorbed from the glass, uncoated and C3-silica, and diatomite, and more than 90% had entered the aqueous phase by 240 h (Table 1). Desorption was slower, and the extents of desorption were less from C2-diatomite and nonporous polystyrene. Little was desorbed from C18-silica and porous polystyrene beads, however, even after 240 h.

**Mineralization.** The rates of degradation of phenanthrene sorbed to the particles were determined in the initial 24 h of the incubation period, except that the test period was 20 h with porous silica or with no beads. The extent of mineralization was determined at 150 h. Phenanthrene was rapidly and extensively degraded in the absence of particles and in the presence of nonporous glass beads, the three types of porous silica, the two types of diatomite, and C3- and C18-silica beads (Table 2). The rates and extents of mineralization in the absence of beads or in the presence of glass and uncoated silica particles were not significantly different, except the extent of mineralization in the presence of silica beads with 15-nm pores was somewhat lower. The rates were somewhat slower in the presence of C3- and C18-coated silica beads containing 6-nm pores and when diatomite particles were present. The data also show that, even though most of the substrate was sorbed by C18-silica and C2-diatomite, it was still readily available.

Biodegradation of phenanthrene was also measured in the presence of nonporous and porous polystyrene beads. Although nonporous polystyrene beads were found earlier to bind most of the compound, that sorbed phenanthrene was readily available to the bacterium, and the compound was quickly and extensively degraded (Table 2). On the other hand, when sorbed to polystyrene with nanopores, little was metabolized. The bioavailability of phenanthrene was especially low when sorbed to beads with 5-nm pores.

A comparison of the data in the two tables shows that, although 35−58% of phenanthrene was sorbed by the uncoated silica, diatomite, and C3-silica beads, phenanthrene was readily degraded. This presumably results from its rapid desorption. Similarly, although 77−94% of the hydrocarbon was sorbed by C2-diatomite and nonporous polystyrene, the desorption rate probably accounts for the great extent of mineralization. On the other hand, the absence of appreciable degradation in the presence of porous polystyrene coincides with the extensive sorption and little desorption of the compound. The data with C18-silica are unexpected, however, because little of the extensively bound compound was desorbed, yet the compound was quickly and extensively metabolized.

## Discussion

Five types of beads were used: glass beads without pores, silica beads with nanopores, beads with hydrophobic surfaces but no pores (polystyrene), diatomite beads with comparatively large (5.4-$\mu$m) pores, and beads with hydrophobic surfaces and nanopores. The nanopores are all smaller than the dimensions of the test bacterium, whose dimensions are ca. 1.5 × 0.9 $\mu$m.

The rates of biodegradation in the presence of beads without hydrophobic surfaces, whether they did or did not contain nanopores, were essentially the same as in particle-free solution. The ready bioavailability of the substrate in the presence of silica beads containing nanopores is consistent with the finding that the internal surfaces of such beads sorb little or no phenanthrene (*15*). This high degree of bioavailability is probably attributable to the low extent of sorption and a rapid rate of mass transfer of the compound from the surface of these particles to the solution phase. In addition, the bacterium readily degraded phenanthrene initially sorbed to beads with hydrophobic surfaces but no pores (polystyrene). The mineralization data suggest that neither hydrophobicity nor surface area alone renders phenanthrene unavailable to bacteria. The microorganism also quickly metabolized phenanthrene initially sorbed to hydrophobically coated beads with nanopores (C3- and C18-silica). This was true despite the sorption of >94% of the substrate by C18-silica and little spontaneous desorption from these particles. The rapid biodegradation of phenanthrene sorbed to C18-silica may be explained by the lack of entry of the substrate into the 6-nm pores, because the length of the octadecyl chain is ca. 2.5 nm (*16*). Instead, the hydrocarbon may be associated only with the external surfaces (*17*). The rapid biodegradation then could be the result of acquisition of the substrate by direct contact of cells with the solid or desorption facilitated by a surfactant produced by the microorganisms (*18, 19*). Dimethyldichlorosilane-bonded diatomite, which contained 5.4-$\mu$m pores, also did not greatly affect the rate and extent of mineralization, but phenanthrene was desorbed from these particles at a reasonable rate.

In marked contrast, little biodegradation occurred when phenanthrene was sorbed to hydrophobic beads made of polystyrene that contained 5- or 300−400-nm pores. More than 97% of the compound had been sorbed in 15 h, and <1.0 and <3.4% had desorbed in 5 and 240 h, respectively. These data thus suggest that bioavailability of a hydrophobic compound such as phenanthrene can be markedly reduced by particles bearing nanopores having hydrophobic surfaces and that nanopores with no such surfaces or particles without nanopores do not reduce bioavailability. The findings are therefore consistent with the hypothesis that hydrophobic compounds may become sequestered and less available to living organisms if those molecules penetrate these porous materials (*1, 20*). Nevertheless, the findings with model solids, such as those used in this investigation, do not confirm that sequestration in natural environments occurs by this mechanism, or this mechanism alone, but they do indicate that the mechanism is plausible. Similar hypotheses have been proposed to explain the persistence of humic matter in soils and sediments. Organic molecules also may be sequestered within the lattice of clay minerals (*21, 22*). Thus, it has been suggested that the resistance of soil organic matter to decomposition arises because that organic matter resides within small pores, where the humic materials are physically inaccessible to microorganisms (*23, 24*). In addition, Mayer (*25, 26*) proposed that organic matter in marine sediments is protected by its location inside pores too small to allow for the entrance or functioning of hydrolytic enzymes.

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