Physical State of Phenanthrene for Utilization by Bacteria

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Experiments were done to determine if bacteria able to utilize phenanthrene as a sole source of carbon obtain the hydrocarbon directly from solid phenanthrene particles suspended in the medium or from the phenanthrene dissolved in the medium. Growth experiments were done in gas-tight fermentors completely filled with air-saturated, mineral salts solution with phenanthrene as the substrate. Generation times were determined by rate of oxygen consumption. Generation times were independent of the amount of solid phenanthrene present, and these generation times were the same as the generation time on medium containing only dissolved phenanthrene. It was concluded that bacteria utilize phenanthrene in the dissolved state.

Wodzinski and Johnson (7) have shown that for naphthalene, phenanthrene, anthracene, and naphthacene, with water solubilities of 98, 9.0, 0.45, and 0.0066 mM, respectively (4), the growth rates of bacteria utilizing these hydrocarbons as sole sources of carbon are related to the water solubilities of these hydrocarbons. The generation times on naphthalene, phenanthrene, and anthracene were found to be 1.5, 10.5, and 29 h, respectively. Attempts at isolating organisms that could grow on naphthalene, the most insoluble hydrocarbon studied, were unsuccessful. Since the cultures grew more slowly on more insoluble hydrocarbons, it is reasonable to assume that the water solubility of the aromatic hydrocarbon may be an important factor in determining the rate of utilization. If this hypothesis is correct, one would predict that very insoluble aromatic hydrocarbons would not be readily utilized.

If the solubility of the aromatic hydrocarbon directly affects the rate of utilization, the bacteria must utilize the dissolved hydrocarbon, and not the solid particles directly, by growing at the interface of the aqueous medium and solid hydrocarbon. Rogoff has shown that bacteria can absorb naphthalene and phenanthrene from saturated solutions of these hydrocarbons (5). Wodzinski and Bertolini have shown that for a naphthalene isolate, grown on naphthalene, the growth rate of the bacteria is independent of the amount of solid naphthalene present in the medium, and this rate of growth is the same as the rate of growth of the isolate on a medium containing only dissolved naphthalene (6). It was concluded that naphthalene is utilized in the dissolved state. The evidence supports the hypothesis that solubility is an important factor in determining the rate of utilization.

Although there is an apparent relation between the solubility of naphthalene, phenanthrene, and anthracene and rate of growth of bacteria on the hydrocarbons and evidence that naphthalene is utilized in the dissolved state, it is not certain that phenanthrene and anthracene are also utilized in the dissolved state. It is possible that the slower growth rates on phenanthrene and anthracene are the result of a chemical structure that is degraded more slowly. It is also possible that, since phenanthrene and anthracene are more insoluble in water than naphthalene, the bacteria may obtain hydrocarbon directly from solid hydrocarbon particles suspended in the medium.

The purpose of this investigation was to obtain direct evidence of whether bacteria utilize phenanthrene in the dissolved or solid state. Such information on phenanthrene would be valuable in evaluating the hypothesis that the solubility of aromatic hydrocarbons is an important factor in determining the rate of utilization by bacteria.

An attempt was made to compare growth rates of bacteria on a medium containing only dissolved phenanthrene with growth rates on a medium containing both dissolved and solid phenanthrene. An attempt was also made to determine the effect of the amount of solid phenanthrene present in the medium on the growth rate of bacteria.
MATERIALS AND METHODS

**Media.** A buffered mineral salts (BMS) solution (7) at pH 7.0 was the basis of the media used in this work. Phenanthrene-saturated medium for experiments with only dissolved phenanthrene present was prepared by adding 1.0 g of phenanthrene to one liter of BMS followed by autoclaving and subsequent standing for at least 1 week at 30 C. Solid hydrocarbon was removed just prior to use by filtering through Schleicher and Schuell no. 588 filter paper. Media for experiments with both dissolved and solid phenanthrene present were prepared by adding 0.5, 0.05, and 0.01 g of powdered, sterile phenanthrene to 500 ml of sterile BMS solution. The particles of phenanthrene were less than 0.1 mm in diameter. To make “purified” BMS-phenanthrene medium, solid phenanthrene was removed from the medium by filtration, and the solid phenanthrene was used to prepare new medium as previously described. This procedure was repeated twice to insure removal of any water-soluble impurities. All media, equilibrated without agitation in a 30 C incubator for 1 week, became saturated with air, as indicated by no increase in dissolved oxygen concentration in the medium after sparging the medium for 15 min at a rate of 500 ml of air per min. Assuming air pressure of 735 mm of Hg, water vapor pressure of 31.82 mm of Hg, and absorption coefficient of oxygen of 0.02608, it was calculated that one liter of air-saturated medium contained 2.16 x 10^-4 mol of oxygen.

**Fermentor.** A 500-ml, gas-tight fermentor for growing cells in the presence of both solid and dissolved phenanthrene was constructed from a 500-ml Erlenmeyer flask fitted with a silicone rubber stopper. The stopper held an oxygen probe (1) and an exit port, which consisted of a piece of glass tubing that passed through the stopper and was itself sealed by a cork covered with Teflon tape. The fermentors were completely filled with air-saturated medium so there was no gas space in the flask. Fermentations were done at 30 C with agitation provided by a cylindrical magnetic stir bar (1 by 3 cm) rotating at 240 rpm. The fermentor for the growing of cells in the presence of only dissolved phenanthrene was exactly like the fermentor described above, except that the stopper held no oxygen probe or exit port and there was a small gas space of less than 10 ml above the medium. Fermentations were done at 30 C with agitation provided by a cylindrical stir bar (1 by 3 cm) rotating at approximately 240 rpm.

**Measurement of oxygen.** The generation times in the experiments with both solid and dissolved phenanthrene present were calculated from the straight line obtained from a plot of the logarithm of the decrease in percentage of oxygen saturation versus time. An oxygen probe (1) was used to measure changes in dissolved oxygen concentration during the fermentation. The oxygen-probe current, which is proportional to oxygen concentration, was observed by measuring the potential across an external resistance of less than 1,500 ohms. The potential was measured by a 10-mV recorder. It was determined that, for the oxygen probes used under the conditions of the experiments, the potential across the external resistance is proportional to oxygen concentration (6).

**Plate counts.** The generation times for experiments using only dissolved phenanthrene were calculated from the straight line obtained from a plot of the logarithm of the number of organisms/ml, determined by plate count, versus time. Samples (1 ml) of culture were removed from the fermentor at 0, 6, 8, 9, 10, 11, and 12 h after inoculation. Samples were then diluted by a factor of 10^4 and 10^5 before plating on nutrient agar. The plates were incubated at 30 C for 48 h before counting.

**Inoculum.** The inoculum for the fermentations, a pure culture obtained from sewage by enrichment culture on phenanthrene, was grown in 500-ml Erlenmeyer flasks containing 200 ml of BMS solution and 0.5 g of phenanthrene. After 24 to 48 h of incubation at 30 C with agitation, the solid phenanthrene was removed by filtration. Some cells were retained by the filter, but a sufficient number of cells was in the filtrate to serve as an inoculum. The filtrate was diluted to 1/20 with distilled water. In fermentations with both solid and dissolved phenanthrene present, 0.1 ml of undiluted filtrate was used. It was determined that the undiluted inoculum contained approximately 4.5 x 10^3 organisms/ml.

**Determination of the presence of solid hydrocarbon in medium saturated with phenanthrene.** The procedure used to determine that the phenanthrene-saturated medium was free of solid phenanthrene after filtering was as follows. The ultraviolet absorption of a filtered, phenanthrene-saturated BMS medium and the same medium diluted to one-half strength were determined. After 0.5-h equilibration in capped tubes, the absorption of the solutions was determined at a wave length of 251 nm. If no solid phenanthrene was present in the filtered, saturated solution, the absorption of the diluted medium should be one-half of the absorption of the undiluted medium.

**RESULTS**

**Isolate.** An isolate able to use phenanthrene as its sole source of carbon was obtained from sewage by enrichment culture. The isolate was a gram-negative rod, motile with polar flagella, which did not produce acid or gas on lactose broth, and reduced nitrate to nitrite. In contrast to the organism isolated by Wodzinski and Johnson (7), this organism was unable to use naphthalene as a sole carbon source. The isolate was tentatively classified as a species of *Pseudomonas*.

**Detection of solid phenanthrene in phenanthrene-saturated BMS medium.** Experiments to determine the presence of solid phenanthrene in the filtered, phenanthrene-saturated BMS medium were negative. The ultraviolet absorption of the filtered, phenanthrene
BMS medium, diluted to half, was 46% of the absorption determined for the undiluted phenanthrene-saturated BMS medium. These are the results expected if no solid phenanthrene was present in the filtered, phenanthrene-saturated BMS medium.

**Oxygen-probe stability.** An oxygen probe in a fermentor filled with air-saturated BMS solution showed a 2% decrease in dissolved oxygen concentration in 30 h. This decrease was probably due to probe output variation over the 30-h period. Thus, the oxygen probes used in this work were stable.

**Growth on solid phenanthrene.** The generation times of the phenanthrene isolate on media containing various amounts of solid phenanthrene are shown in Table 1. Exponential growth was observed in all experiments. At least 60% of the decrease in percentage of oxygen concentration fitted a straight line obtained in the plot of the logarithm of the percentage of decrease in oxygen saturation versus time. The generation times determined from the fermentations with various amounts of phenanthrene did not vary with the amount of solid present in the medium. The phenanthrene did not clump nor did the cells appear to accumulate around phenanthrene particles during the fermentation. In all experiments with solid phenanthrene present, growth stopped due to a lack of dissolved oxygen at the end of the fermentation.

In fermentations without substrate, there was a 10% uptake of dissolved oxygen in 12 h. This utilization could be due to growth on metabolizable impurities in the system, material stored by the inoculum, or phenanthrene carried over with the inoculum.

In experiments with higher concentrations of solid phenanthrene in the medium, the observed growth could possibly be due to water-soluble impurities in the substrate. For example, assuming that the phenanthrene contains 2% impurity, a flask containing 0.5 g of phenanthrene would contain 10 mg of impurity. Assuming a 40% cell yield, growth on this amount of impurity would utilize seven times the amount of available dissolved oxygen. However, the generation time of the phenanthrene isolate on "purified" phenanthrene-BMS medium was the same as the generation time on regular medium. It is assumed that any water-soluble impurities would have been removed by the purification procedure. Also, the generation times of the phenanthrene isolate on very small amounts of phenanthrene were identical to generation times on the large amounts of phenanthrene, and the small amount of phenanthrene could not contain sufficient impurity to cause the observed growth. For example, a flask containing 0.01 g of phenanthrene could contain 0.2 mg of impurity. Assuming a 40% cell yield, growth on this amount of impurity would use only 15% of the available dissolved oxygen. Since there was a 100% oxygen uptake in all experiments, the growth observed could not be due to impurities in the substrate.

**Growth on dissolved phenanthrene.** The generation times of the phenanthrene isolate on BMS solution containing only dissolved phenanthrene, obtained by plate count, were 1.3, 1.3, and 1.2 h. Exponential growth was observed in each experiment. The numbers of organisms counted in samples from 6 to 12 h fitted a straight line obtained in the plot of the logarithm of the number of organisms/ml versus time. The generation times determined from the fermentations with dissolved phenanthrene were identical to generation times of the phenanthrene isolate in fermentations with both solid and dissolved phenanthrene. After 12 h, when the experiment was terminated, the cells were still growing exponentially.

In fermentations with only dissolved phenanthrene present, there was an increase of 240 × 10^4 bacteria/ml from 6 to 12 h. In control fermentations without substrate, there was an increase of 30 × 10^4 bacteria/ml from 6 to 12 h. Thus, about 10% of the observed growth must be due to impurities in the system.

**Purity of phenanthrene used.** Since Wodzinski and Johnson (7) reported a generation time of 10.5 h for an isolate on phenanthrene, the 1.3-h generation time of the phenanthrene isolate used in this study, in the fermentations using dissolved and solid phenanthrene media, raised some question as to whether the substrate was actually phenanthrene and also as to its relative purity. Therefore, the ultraviolet spectrum of 0.08 mg of phenanthrene in 100 ml of cyclohexane was taken in a 1-cm cell on the Cary 14 spectrophotometer. The scan was between 220 and 350 nm. From the absorbance of the major peak, 0.288, and the molar concentration of the phenanthrene solution, 4.75 × 10^-6 M, the extinction coefficient was calcu-

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**Table 1. Generation times of a phenanthrene isolate grown on media containing both dissolved and solid phenanthrene**

| Initial amount of solid phenanthrene (g/liter) | No. of trials | Mean generation time ± s (h) |
|---------------------------------------------|--------------|------------------------------|
| 1.00                                        | 9            | 1.3 ± .02                    |
| 1.00 purified                               | 2            | 1.4, 1.4                     |
| 0.10                                        | 8            | 1.3 ± .10                    |
| 0.02                                        | 7            | 1.3 ± .05                    |
lulated to be $6.1 \times 10^4$. A previously published extinction coefficient was given as $6.31 \times 10^4$ (3). The only peaks observed in the absorption spectrum were at 251, 277, 283, and 294 nm compared with the major peaks of the published spectrum at 253, 275, 282, and 293 nm (3). The ultraviolet absorption spectrum obtained and the extinction coefficient calculated demonstrated that the substance was phenanthrene in a relatively pure state.

**DISCUSSION**

Since the phenanthrene isolate grew on BMS solution saturated with phenanthrene at the same rate that it grew on BMS solution with both dissolved and solid phenanthrene, the isolate growing in the medium containing both dissolved and solid phenanthrene probably utilized dissolved phenanthrene. Utilization of dissolved phenanthrene is also indicated by the fact that the amount of solid phenanthrene present in the medium did not affect the rate of growth. Dunn has proposed an interfacial kinetic model that predicts the growth kinetics for an organism growing at an interface (2). The model assumes that absorption and desorption rates from an interface are fast compared with the rate of growth. For a small population, growing at an interface (utilizing solid phenanthrene directly), the model predicts dependence of the growth rate on interfacial surface area. If the cells are utilizing dissolved phenanthrene, as long as the concentration of dissolved phenanthrene is not rate-limiting or the rate of phenanthrene transfer from the solid to the aqueous phase is more rapid than the rate of utilization, the population will grow exponentially and the rate will not be dependent on the surface area of the solid. The results found in these experiments show that, for growth of the phenanthrene isolate in the presence of solid phenanthrene, the rate of growth is exponential and independent of the surface area, which indicates utilization of the dissolved phenanthrene and not of the solid directly. In the fermentations with both solid and dissolved phenanthrene present, the organisms did not appear to accumulate around solid particles of phenanthrene.

Compared with the generation time of 10.5 h determined by Wodzinski and Johnson (7), the 1.3-h generation time found in these experiments is very fast. The organism isolated in the experiments is a different organism, which may have a greater ability to use phenanthrene than the isolate in the earlier work. The difference in isolates is demonstrated by the fact that the isolate used in this work did not grow on naphthalene.

The results show that, when phenanthrene is the sole carbon source for growth, bacteria utilize dissolved phenanthrene rather than the solid particles. It is obvious from the generation times obtained in this work, compared with those determined by Wodzinski and Johnson (7), that the growth rate of bacteria able to utilize aromatic hydrocarbons cannot be accurately predicted solely on the basis of the solubility of the aromatic hydrocarbon; cultures differ too much in the rate at which they can utilize a particular aromatic hydrocarbon.

Finding that phenanthrene is utilized in the dissolved state supports the hypothesis that polynuclear aromatics are utilized in the dissolved state. If other polynuclear aromatic hydrocarbons are utilized in the dissolved state, as are naphthalene (6) and phenanthrene, it may be possible to predict that a very insoluble aromatic hydrocarbon would not be utilized readily by bacteria because the substrate would not be available to the bacteria. If the hypothesis is correct, one would also predict that when the rate of aromatic hydrocarbon utilization exceeds the rate of hydrocarbon transfer from the solid to the aqueous phase, as is likely to be the case with dense populations or very insoluble hydrocarbons, the rate of growth will be linear and a function of the rate of hydrocarbon transfer from the solid to the aqueous phase.

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