Physical State in Which Naphthalene and Bibenzyl are Utilized by Bacteria

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The generation times of a strain of Pseudomonas grown on a mineral salts medium in the presence of various amounts of naphthalene did not vary with the amount of solid present, and these generation times were the same as the generation time on mineral salts medium containing only dissolved naphthalene. The generation time of a soil isolate grown on mineral salts medium in the presence of 0.5 g of solid bibenzyl per liter was the same as the generation time on a mineral salts medium saturated with bibenzyl. The evidence indicates that naphthalene and bibenzyl are utilized in the dissolved state.

Wodzinski and Johnson have presented data showing that the growth rates of bacteria on naphthalene, phenanthrene, and anthracene are related to the respective solubilities of these aromatic hydrocarbons in water (6). The greater the solubility of the hydrocarbon, the faster the growth rate. Attempts at isolating organisms that could grow on naphthalene, the most insoluble hydrocarbon studied, were unsuccessful. The solubilities of naphthalene, phenanthrene, anthracene, and naphthacene are 98, 9.0, 0.45, and 0.066 μM, respectively (3). Rogoff has shown that bacteria can absorb naphthalene and phenanthrene from aqueous solution (5). These data seem to suggest that aromatic hydrocarbons are utilized in the dissolved state.

If bacteria have enzymatic systems to degrade a given hydrocarbon, the solubility of the hydrocarbon could determine the availability of the hydrocarbon to the bacteria, and the availability of the hydrocarbon could control the rate of utilization. Thus, if bacteria do utilize dissolved aromatic hydrocarbon as a sole source of carbon, one could predict that a very insoluble solid aromatic hydrocarbon would not readily be utilized, regardless of any enzymatic potential the cell may have.

Although for naphthalene, phenanthrene, and anthracene there is a relationship between growth rate and solubility, the rate of growth may be determined, not by the solubility of the hydrocarbon, but by the chemical structure of the hydrocarbon. The bacteria may obtain hydrocarbons directly from the solid phase, and the greater the number of rings in the hydrocarbon, the more difficult it is for the bacteria to utilize the aromatic hydrocarbon.

The purpose of this investigation was to obtain direct evidence to determine whether bacteria obtain aromatic hydrocarbons from aqueous solution or directly from the solid phase.

An attempt was made to compare growth rates of bacteria on media containing dissolved hydrocarbon to growth rates on media containing both dissolved and solid hydrocarbons. An attempt was also made to determine the effect of the amount of solid hydrocarbon present in the media on growth rate. Naphthalene and bibenzyl were studied because they are relatively soluble in water and readily utilized.

MATERIALS AND METHODS

Media. A buffered mineral salts medium (BMS) at pH 7.0 (6) was the basic medium used in this work. Solutions saturated with naphthalene and bibenzyl were prepared by adding 1.0 g of naphthalene or 0.50 g of bibenzyl, respectively, to 1 liter of BMS, followed by autoclaving and subsequent standing for at least 1 week at 30°C before use. Solid hydrocarbon was removed prior to use by filtering through Schleicher and Schuell no. 588 filter paper. To make "purified" BMS solutions saturated with hydrocarbon, the excess solid removed by filtration was used to make a BMS solution saturated with hydrocarbon as previously described. It was assumed that any water-soluble impurities, if present, would be removed in the first filtration. Media with solid naphthalene or bibenzyl were prepared by adding powdered hydrocarbon to sterile BMS medium. After sterilization, the medium was saturated with air at
30 C. 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 1 liter of medium contained \(2.16 \times 10^{-4}\) moles of oxygen.

**Fermentor.** A 500-ml gas-tight fermentor was constructed from a 500-ml Erlenmeyer flask fitted with a silicone rubber stopper. The silicone rubber stopper held an oxygen probe (1) and an exit port, which consisted of a piece of glass tubing that passed through the stopper. The exit port could be closed by inserting a short piece of rubber tubing and a screw clamp after filling the fermentor. The fermentors were completely filled with air-saturated medium so that there was no gas space in the flask. The fermentations were done in an incubator at 30 C, and agitation was provided by a cylindrical stir bar (1 by 5 cm) rotating at 180 rev/min. The stir bar was rotated by means of a magnetic stirring motor. The generation times were calculated from the straight line obtained from a plot of the logarithm of the decrease in percent oxygen saturation versus time.

**Measurement of oxygen.** An oxygen probe was used to measure changes in dissolved oxygen 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 with a 10 mv recorder. The potential across the external resistance should be proportional to oxygen concentration. To check this, 500 ml of BMS was sparged with various mixtures of air and nitrogen at 25 C at a rate of 275 ml of gas per min. The composition of the mixture was controlled by regulating the flow rates of the air and nitrogen. The BMS was agitated by means of a magnetic stir bar rotating at 400 rev/min.

**Inoculum.** The inoculum for bibenzyl fermentations was grown in 500-ml Erlenmeyer flasks containing 60 ml of BMS and 0.20 g of bibenzyl. After 20 hr of incubation, the solid bibenzyl was removed from the medium by filtration. Some cells were retained by the filter, but a sufficient amount of cells was in the filtrate to serve as an inoculum. The filtrate was diluted by 1/20 with distilled water. In all bibenzyl fermentations, 0.1 ml of the dilute filtrate was used to inoculate the fermentor. It was determined that inoculum contained approximately 3.0 \(\times \) \(10^{-4}\) g of cells.

The inoculum for naphthalene fermentations was prepared by plating the organism on BMS agar in inverted petri dishes. The carbon source was naphthalene vapor derived from crystals placed on the inside of the cover. After 20 hr, several loops of growth were transferred to 10 ml of distilled water and filtered. Some cells were retained by the filter, but a sufficient amount of cells was in the filtrate to serve as an inoculum. The filtrate was diluted by 1/20 with distilled water. In all naphthalene fermentations, 0.1 ml of dilute filtrate served as the inoculum. It was determined that the inoculum used contained approximately 6.0 \(\times \) \(10^{-4}\) g of cells.

**Determination of the presence of solid hydrocarbon in solutions saturated with hydrocarbon.** The following procedure was used to insure that the saturated solutions of hydrocarbon were free of solid hydrocarbon. The ultraviolet absorption of a solution saturated with hydrocarbon and a solution saturated with hydrocarbon diluted to half-strength were determined. Absorption of naphthalene solutions were done at a wavelength of 275 nm. Absorption of bibenzyl solutions was done at a wavelength of 253 nm. For bibenzyl solutions, a 10-cm quartz cuvette was used. If no solid was present in the solution saturated with hydrocarbon, the absorption of the diluted solution should be half the absorption of the solution saturated with hydrocarbon.

**Organism.** The organism used for the studies with naphthalene was of the genus *Pseudomonas* and has been described previously (6). For the studies on bibenzyl, an isolate obtained from soil by enrichment culture was used.

**RESULTS**

**Isolates.** An isolate able to utilize bibenzyl as its sole source of carbon was isolated by enrichment culture. The bibenzyl isolate was a gram-negative rod; was not motile; grew on BMS medium with simple carbon sources such as glucose, gluconate, and ethanol; and did not produce acid or gas on lactose broth.

**BMS solutions saturated with hydrocarbon.** Experiments to detect the presence of solid hydrocarbon in filtered solutions saturated with hydrocarbon were negative. The ultraviolet absorption of the BMS medium saturated with naphthalene and diluted to half-strength with BMS medium was 49 to 51% the absorption of the BMS solution saturated with naphthalene. The ultraviolet absorption of water saturated with bibenzyl and diluted to half with water was 42 to 44% the absorption of water saturated with bibenzyl. Water saturated with bibenzyl was prepared the same way as BMS medium saturated with bibenzyl. The results obtained are the results expected if no solid was present in the saturated solutions.

**Solubility of bibenzyl in water at 30 C.** The absorbance \((\lambda_{\text{max}} = 253 \text{ nm})\) of water saturated with bibenzyl, determined in a 10-cm quartz cuvette, was 0.349. The absorbance \((\lambda_{\text{max}} = 258)\) of bibenzyl in ethanol \((5.5 \times 10^{-4}\) M), determined in a 1-cm quartz cuvette, was 0.243. The \(\epsilon_{\text{max}}\) of bibenzyl in ethanol was calculated to be 442. Assuming that \(\epsilon_{\text{max}}\) of bibenzyl in water was also 442, it was calculated that the solubility of bibenzyl in water was 0.014 g/liter.

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

**Measurement of oxygen.** The dissolved oxygen was proportional to the oxygen-probe current observed as a potential across an external resistance. The data are shown in Table 1.

**Growth on naphthalene.** The generation times of the naphthalene isolate grown on dissolved naphthalene and on various amounts of solid naphthalene are shown in Table 2. Exponential growth was observed in all experiments. At least 70% of the decrease in percent oxygen fitted a straight line when the logarithm of the percent decrease in oxygen versus time was plotted. In all naphthalene experiments, a lack of dissolved oxygen limited growth at the end of the fermentation. The generation times obtained in the presence of various amounts of naphthalene did not vary with the amount of solid present, and the generation times in the presence of solid were the same as the generation time on BMS containing only dissolved naphthalene. The powdered naphthalene did not clump during the fermentation.

When fermentations were done without substrate, approximately 8% of the dissolved oxygen was utilized in 21 hr. This oxygen uptake could be due to growth on impurities in the system.

In fermentations done on medium saturated with naphthalene in the presence of 1.0 g of solid naphthalene per liter, it was calculated that at the end of the fermentation approximately 0.02 g of naphthalene per liter was utilized for growth. Since the naphthalene is at least 98% pure, 1 g could possibly contain 0.02 g of a metabolizable impurity. The observed growth on this medium could therefore be due to utilization of a water-soluble impurity. However, since identical generation times were obtained on both "purified" and regular medium, the growth obtained on medium saturated with naphthalene could not be due to utilization of an impurity.

**Growth on bibenzyl.** The generation times of the bibenzyl isolate grown on dissolved bibenzyl and on various amounts of solid bibenzyl are shown in Table 3. Exponential growth was observed in all experiments. At least 70% of the decrease in percent oxygen fitted a straight line when the logarithm of the percent decrease in oxygen versus time was plotted. In fermentations where only dissolved bibenzyl was present, a lack of dissolved bibenzyl limited growth at the end of the fermentation and approximately 30% of the dissolved oxygen was depleted at the end of the fermentation. In all fermentations with solid bibenzyl, oxygen limited growth at the end of the fermentation. The powdered bibenzyl did not clump during the fermentation.

When fermentations were done without substrate, approximately 8% of the dissolved oxygen was utilized in 21 hr. This oxygen uptake could be due to growth on impurities in the system.

When bibenzyl isolate was grown in the presence of various amounts of solid bibenzyl, the greater the amount of solid present, the greater the generation time, which would indicate the presence of a water-soluble inhibitor.

**Table 1. Relationship between oxygen concentration and oxygen-probe current measured as a potential across an external resistance**

| Oxygen-probe no. | External resistance (Ω) | Potential with 100% air (mv) | Potential with 51.4% air and 48.6% N₂ (mv) | Potential with 24.1% air and 75.9% N₂ (mv) |
|-----------------|------------------------|-------------------------------|-------------------------------------------|------------------------------------------|
| 1               | 650                    | 9.72                          | 4.99 (5.04)                               | 2.31 (24.1)                              |
| 2               | 650                    | 9.52                          | 4.90 (5.00)                               | 2.26 (2.40)                              |
| 3               | 750                    | 9.68                          | 4.98 (5.02)                               | 2.30 (2.41)                              |

*Expected value, indicated in parentheses, was calculated by multiplying potential at 100% air by percent air in gas mixture.*

**Table 2. Generation times of a naphthalene isolate grown on naphthalene**

| Physical state of naphthalene | Initial amt of solid (g/liter) | No. of trials | Avg generation time ± σ (hr) |
|-------------------------------|--------------------------------|---------------|-----------------------------|
| Dissolved                     | None                           | 8             | 1.2 ± 0.2                   |
| Dissolved (purified)          | None                           | 3             | 1.2 (1.2, 1.2, 1.3)         |
| Solid                         | 0.10                           | 7             | 1.2 ± 0.1                   |
| Solid                         | 1.00                           | 7             | 1.2 ± 0.1                   |
| Solid                         | 4.00                           | 7             | 1.2 ± 0.1                   |

**Table 3. Generation times of a bibenzyl isolate grown on bibenzyl**

| Physical state of bibenzyl    | Initial amt of solid (g/liter) | No. of trials | Avg generation time ± σ (hr) |
|-------------------------------|--------------------------------|---------------|-----------------------------|
| Dissolved                     | None                           | 9             | 2.0 ± 0.2                   |
| Dissolved (purified)          | None                           | 2             | 1.3 ± 1.4                   |
| Solid                         | 0.05                           | 8             | 1.5 ± 0.2                   |
| Solid                         | 0.50                           | 9             | 1.9 ± 0.2                   |
| Solid                         | 2.00                           | 7             | 3.0 ± 1.0                   |

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In fermentations done on media saturated with bibenzyl, the substrate limited growth at the end of the fermentation. If the growth were due to utilization of a water-soluble impurity and all of the impurity dissolved into the water, there would be no growth on the "purified" medium. The rate of growth on the "purified" medium saturated with bibenzyl was slightly faster than on the regular medium saturated with bibenzyl, which would indicate that growth was not due to an impurity. The faster rate of growth on the "purified" medium may be due to the removal of an inhibitor.

The generation time obtained in the presence of 0.5 g of solid bibenzyl per liter was the same as the generation time on a solution saturated with bibenzyl, which was saturated in the presence of 0.5 g of bibenzyl per liter before the fermentation.

**DISCUSSION**

The data in Table 2 provide strong evidence that the naphthalene isolate utilized dissolved naphthalene and did not utilize the solid directly. The rate of growth on the BMS solution saturated with naphthalene was the same as the rate of growth in the presence of solid. Since the generation times are the same, the isolate growing in the presence of solid naphthalene probably utilized dissolved naphthalene. Utilization of dissolved naphthalene is also indicated by the fact that the amount of solid present 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 the interface are fast compared to growth. For a small population, growing at an interface (utilizing solid directly), the model predicts dependence of the growth rate on interfacial surface area. If the population is large and the interfacial surface area is small, the model predicts that the rate of growth will be linear. If the cells are utilizing dissolved naphthalene, as long as the concentration of dissolved naphthalene is not rate-limiting or the rate of naphthalene 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. Table 2 shows that for growth of the naphthalene isolate in the presence of solid naphthalene, the rate of growth is exponential and independent of the surface area, which indicates utilization of the dissolved naphthalene and not the solid directly.

When bibenzyl isolate was grown in the presence of various amounts of bibenzyl, it was found that the greater the amount of solid bibenzyl, the greater the generation time. This relationship is not due to the variation in surface area of the solid; the opposite relationship would be observed if cells were growing at the interface. The relationship between amount of bibenzyl and growth rate can be explained by assuming that an inhibitor is present. The greater the amount of bibenzyl, the greater the amount of inhibitor. The presence of an inhibitor is also indicated by the fact that cells grown on a "purified" BMS solution saturated with bibenzyl grew faster than on regular BMS solution saturated with bibenzyl. Some or all of the inhibitor would be removed in the purification. The fermentations on various amounts of solid bibenzyl cannot provide an insight into the form in which the hydrocarbon is utilized; however, the data do support the hypothesis that the cells utilize dissolved bibenzyl. Utilization of dissolved bibenzyl is indicated by the fact that the same generation times were obtained in the presence of 0.5 g of solid bibenzyl and in BMS saturated with bibenzyl, which was saturated in the presence of 0.5 g of bibenzyl. Any inhibitor present would be equal in the two systems and the effect of the inhibitor on the rate of growth would be the same. Since the rates of growth are equal, it would seem that the organisms, growing in the presence of solid bibenzyl, are using dissolved bibenzyl.

The data show that the bacteria used in the study utilize dissolved naphthalene and bibenzyl and do not utilize the solid phase directly. This evidence supports the hypothesis that the rate of utilization of solid aromatic hydrocarbons is strongly influenced by the solubility of the hydrocarbon, which determines the availability of the hydrocarbon to the cell. If the hypothesis is true, for a solid aromatic hydrocarbon it may be possible to predict whether cells would be unable to utilize the compound readily, if the solubility is known, regardless of the enzymatic potential of the cell.

Although the data show the bacteria utilize dissolved naphthalene, the growth rates of cells on phenanthrene and anthracene (6) may not be determined by the solubility of the hydrocarbons. It is possible that with slightly soluble compounds, such as phenanthrene and anthracene, the cells utilize solid hydrocarbon directly, and the slow growth rates are due to slow enzymatic utilization of the compounds or slow uptake of hydrocarbon from the solid.
phase. It may also be possible to have an accommodation (4) of the hydrocarbon in the aqueous phase. However, the fact that for naphthalene, phenanthrene, and anthracene the rate of growth is related to the solubility of the compounds and dissolved naphthalene is utilized by cells makes more tenable the hypothesis that the solubility of the hydrocarbon (availability) is of importance to the ability of bacteria to utilize solid aromatic hydrocarbons rapidly.

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