THE MICROBIOLOGY OF COAL

I. Bacterial Oxidation of Phenanthrene

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The simultaneous addition of phosphate with 2,4-D enhances the toxicity of the latter to *A. vinelandii*. This enhanced toxicity is related to the magnesium concentration of the environment.

The microbiology of coal has received little attention by comparison with the large amount of research on petroleum. Coal is a much more complex organic material than petroleum and probably consists of condensed rings of an aromatic and hydroaromatic nature. The ring systems in coal resist microbial oxidation; only the highly oxidized coals give any indication of supporting an indigenous microflora (Lieske and Hofmann 1928). For this reason a study of the microbial attack on polynuclear hydrocarbons was initiated as a means of investigating the constitution of coal.

The higher polynuclear hydrocarbons serve as model compounds in determining the products of microbial oxidation of highly condensed ring systems. Coal contains phenolic-hydroxyl and is thought to contain quinone and carboxyl groups. The demonstration that these substituent groupings appear in the intermediate products of polynuclear hydrocarbon oxidation may help to explain their presence in coal. Study of these compounds should also aid in determining the effects of steric configuration, substitution, size of the molecule and other physical and chemical factors on the resistance of the compounds to microbial attack. It may be possible to draw analogies from such studies to explain the relative resistance of a material like coal to microbial attack.

We have chosen phenanthrene as one such model compound. The attack of phenanthrene by soil bacteria has been reported by Tausson (1928*b*, 1929), who also described its utilization by certain anaerobic sulfate-reducing bacteria (Tausson and Vesselov, 1934). In an earlier study Tausson (1928*a*) described two new species, *Bacillus phenanthrenicus bakiensis* and *B. phenanthrenicus guricus*, which in pure culture could assimilate phenanthrene. Sisler and ZoBell (1947) noted that marine bacteria assimilated phenanthrene and anthracene more rapidly than naphthalene, or the higher homologues 1,2-benzanthracene and 1,2,5,6-dibenzanthracene. No intermediates in the oxidations were isolated in these studies; naphthalene is the largest polynuclear hydrocarbon whose oxidation has been studied in detail. The present experiments were initiated to study the dissimilation of phenanthrene by pure cultures of bacteria, and to determine the intermediate products in the oxidative pathway.

MATERIALS AND METHODS

Pure cultures of bacteria capable of utilizing phenanthrene as a sole carbon source were isolated using a soil enrichment technique. The enrichment was carried out in 500-ml Erlenmeyer flasks containing 100 ml of a medium of the following composition: phenanthrene, 1.0 per cent; NH_4O_3, 0.25 per cent; $MgSO_4$, 0.05 per cent; $MnCl_2 \cdot 4H_2O$, 0.02 per cent; $FeCl_2 \cdot 4H_2O$, 0.005 per cent; $CaCl_2$, 0.005 per cent; K_2HPO_4, 0.10 per cent and KH_2PO_4, 0.05 per cent. One gram of a garden soil was used as inoculum. The medium was prepared with distilled water as two double strength salt solutions, one of which contained only the two phosphate salts. The phenanthrene and the two salt solutions were sterilized separately by autoclaving at 121 C for 20 min. After cooling, the phenanthrene was ground with a sterile mortar and pestle and combined with the salt solutions. The pH of the medium was 7.0 to 7.15 without adjustment. When an agar medium was desired agar was added at a concentration of 1.5 per cent. After isolation of the cultures, 0.05 per cent of yeast extract (Difco) was routinely added to the medium. Incubation was carried out on a reciprocating type shaker (90 rpm, 3.5 in stroke) at room temperature. All fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium, 4-liter Erlenmeyer flasks containing 1000 ml, or 2800-ml Fernbach type flasks containing 400 ml of medium.

Cultures for manometric experiments. Stock cultures were maintained on 100-ml portions of the medium in 500-ml Erlenmeyer flasks. These were incubated for 72 hr on the shaker and stored at 5 C. Transfers were made biweekly. Occasional passage through nutrient broth prevented devitalization of the culture used. Cell crops were obtained by inoculating a 4-liter Erlenmeyer flask containing 1 liter of the salt solution, 0.25 per cent phenanthrene and 0.1 per cent yeast extract (Murphy and Stone, 1955) with 50 ml of a stock culture of the organism. After 48 to 72 hr of incubation, the medium was filtered through glass wool and the cells were removed by centrifugation in the cold. The cells were washed twice with M/50 phosphate buffer, pH 7.38, and resuspended in 10 ml of buffer. The Warburg apparatus was used in the conventional manner to measure oxygen uptake and carbon dioxide evolution (Umbreit, Burris and Stauffer 1949).

Analytical methods. Infrared analyses were carried out in a Perkin-Elmer Model 21 double-beam infrared spectrophotometer. Samples were prepared for analysis as Nujol mulls (a small sample was thoroughly mixed with Nujol to form a thin paste). Ultraviolet analyses were made in a Cary Model 11 ultraviolet spectrophotometer using water or 95 per cent ethyl alcohol as solvent.

Ferric chloride tests were carried out as described by Soloway and Rosen (1953). Quantitative determination of residual phenanthrene was made gravimetrically as follows: The culture medium after fermentation was filtered through a tared, coarse fritted glass filter crucible, which did not retain the bacterial cells. The residue on the filter was exhaustively washed with 0.5 N HCl to remove undissolved salts, and then washed with several small portions of distilled water, the crucible was dried in a vacuum oven for 3 hr at 80 C and reweighed. An uninoculated flask of phenanthrene medium was carried through the same procedure and served as a control.

EXPERIMENTAL RESULTS

Identity of microorganism. After a minimum of five weekly transfers in the enrichment procedure, subcultures were made periodically from the shaken flasks to agar plates on which finely ground phenanthrene had been sprinkled before hardening of the agar. Several cultures were isolated in this manner, one of which was selected as noted below for use throughout these studies. The organism used was a gram-negative motile rod producing small, circular, granular colonies and liquefaction on gelatin plates, and saccate liquefaction with a whitish sediment in gelatin stabs. A ring of green pigment is formed at the top of the liquefied gelatin. Agar colonies were irregular, rugose, glistening, raised and iridescent, a soluble faint green pigment being produced. Growth on agar slants was spreading, rugose and iridescent. Growth in nutrient broth produced turbidity and a pellicle. The organism grew at 37 C, reduced and peptonized litmus milk without coagulation, reduced nitrates to nitrites and produced a small amount of acid from glucose. Sucrose, lactose, maltose and mannitol were not attacked. A bright green pigment was produced in asparagine broth. The organism is a member of the genus *Pseudomonas* and resembles the type species *Pseudomonas aeruginosa*, described in *Bergey's Manual* (Breed, *et al.*, 1948). It bears some resemblance to the naphthalene-attacking *Pseudomonas boreopolis*.

Phenanthrene decomposition. Phenanthrene oxidation was carried out in 500-ml Erlenmeyer flasks, incubation proceeding for 96 hr at which time determinations of residual phenanthrene were made. On the basis of 101 per cent recovery for the control flasks, decomposition varied between 46 and 78 per cent in the different isolates tested. A culture consistently decomposing from 65 to 78 per cent of the phenanthrene provided was chosen for these studies. It was noted that after 36 to 48 hr of incubation, the fermentation liquor became a deep buff color and gave a positive bluish-green ferric chloride test. This indicated the presence of a phenolic compound, possibly a hydroxynaphthoic acid (Soloway and Rosen, 1953).

Isolation and identification of 1-hydroxy-2-naphthoic acid. Each of four Fernbach flasks of culture solution was inoculated with 50 ml of a stock culture. Ferric chloride tests were weakly positive after 40 hr, and strongly positive after 64 hr of incubation. The cultures were pooled and concentrated under reduced pressure to approximately 50 per cent of their volume. The concentrate was acidified with 6 N hydrochloric acid and continuously extracted with ether for 24 hr. The ether was extracted with 10 per cent sodium bicarbonate, and the bicarbonate layer separated and acidified with concentrated hydrochloric acid, giving a white precipitate. Ether

was added and the mixture shaken to dissolve the precipitate. The aqueous layer was extracted twice with fresh portions of ether, and the combined ether layers were washed with saturated sodium chloride solution and dried with anhydrous sodium sulfate. The ether was evaporated under a stream of nitrogen giving 0.84 g of a crystalline material. Recrystallization from benzene-acetic acid yielded 0.46 g of white crystals, m.p. 196.5–197 C. The infrared and ultraviolet spectra were identical with those of an authentic sample of 1-hydroxy-2-naphthoic acid.

Manometric studies with resting cell suspensions. Phenanthrene oxidation was studied in the Warburg apparatus using 5, 10 and 20 μmoles of phenanthrene as the substrate. Phenanthrene was oxidized at all levels of concentration used (figure 1). Similar experiments using phenanthrene-grown cells were made using salicylic acid, catechol, and protocatechuic acid as substrates. The results shown in figure 2 show that the phenanthrene-grown cells possess the enzymes necessary to oxidize all three substrates. Experiments in which 1-hydroxy-2-naphthoic acid was used as substrate indicated that the rate of oxygen uptake in the presence of this compound was considerably less than that of the other compounds. Carbon dioxide evolution, as well as oxygen uptake, was measured with 1-hydroxy-2-naphthoic acid as substrate; the amount of carbon dioxide evolved after 120 min was consistently within the range of from 3.5 to 5.5 μmoles. Ferric chloride tests on the flask contents after the allotted reaction period were positive for flasks in which 10 μmoles of the acid had been used, but negative for flasks in which 5 μmoles had been used.

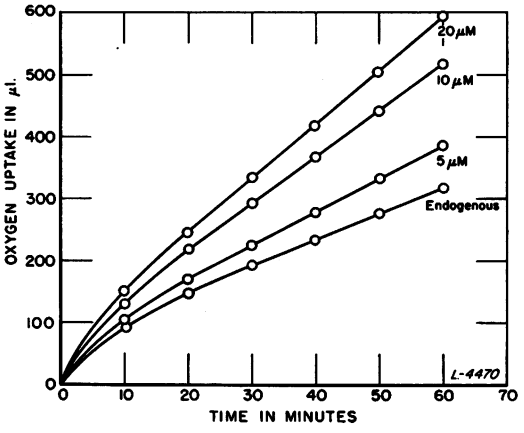

Figure 1. Oxidation of phenanthrene by resting cell suspensions.

Decarboxylation of 1-hydroxy-2-naphthoic acid by the organism might produce 1,2-dihydroxynaphthalene. Murphy and Stone (1955) demonstrated that in the Warburg flask under neutral to alkaline conditions, this compound was oxidized to 1,2-naphthoquinone, even in the absence of cells or extracts. To determine the effects of the quinone on the organism used, experiments

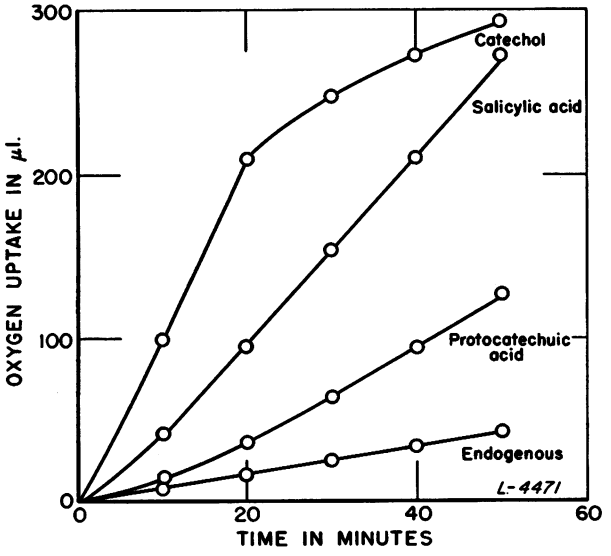

Figure 2. Oxidation of aromatic compounds by resting cell suspensions. Substrate concentration = 5 μ moles.

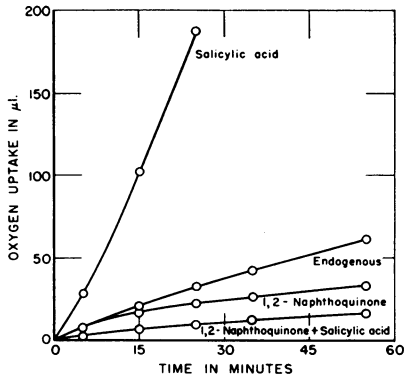

Figure 3. Inhibition of resting cell suspensions by 1,2-naphthoquinone. Substrate concentration = 4 μmoles.

were run using various concentrations of 1,2-naphthoquinone, alone or in combination with other substrates. The data presented in figure 3 show that at a concentration of 4 μmoles, 1,2-naphthoquinone inhibited endogenous respiration and interfered with the oxidation of salicyclic acid. This amount is comparable to the theoretical amount which could be produced from the decarboxylation of the 3.5 to 5.5 μmoles of hydroxynaphthoate observed. Other experiments in which smaller concentrations of the quinone were used as substrate, or were tipped into the flask during oxidation of salicylic acid, showed no inhibitory effects due to the quinone.

DISCUSSION

The organism used in these studies is a member of the genus *Pseudomonas*; it resembles *Pseudomonas aeruginosa* and a poorly defined naphthaline-attacking species *Pseudomonas boreopolis*. It differs from the other hydrocarbon attacking pseudomonads in its proteolytic activities. It is doubtful whether assignment to a new species is necessary on the basis of present data.

The isolate is able to decompose fairly large amounts of phenanthrene in a relatively short time. Tausson (1928*a*) studying phenanthrene oxidation by bacteria isolated from soil ran growth experiments with *o*-hydroxybenzyl alcohol, *o*-hydroxybenzaldehyde, salicylic acid and catechol as substrates. On the basis of the rapidity with which the compounds were assimilated, he postulated primary attack on the phenanthrene molecule at the 9,10-position, with the formation of the above compounds as intermediates (Pathway I, figure 4). The isolation of 1-hydroxy-2-naphthoic acid from our cultures, and the finding that phenanthrene-grown cells of the organism possess the enzymes necessary to oxidize salicylic acid and catechol, indicates oxidation by our culture via end-ring attack (Pathway II, figure 4). The possibility of differences in point of attack due to differences in the metabolic patterns of the organisms used cannot, however, be obviated.

That 1,2-naphthoquinone is produced by the organism from 1,hydroxy-2-naphthoic acid has not been unequivocally demonstrated. However, one line of reasoning points to the possibility that this pathway may exist. At a concentration of 5 μmoles of 1-hydroxy-2-naphthoic acid, the amount of carbon dioxide evolved during oxidation in the Warburg flask is roughly equal to that amount which would be evolved by decarboxylation of the acid present. At concentrations equivalent to those theoretically produced by decarboxylation of hydroxynaphthoate (4 μmoles), 1,2-naphthoquinone inhibits respiration of the cells and interferes with the oxidation of salicylic acid. When 5 μmoles of hydroxynaphthoate are used as substrate, the flask contents after reaction give a negative test with ferric chloride. This indicates that the acid is no longer present, at least in amounts detectable by the test. When an excess of acid is supplied, approximately the same amount of carbon dioxide is evolved as with 5 μmoles, but a positive ferric chloride test

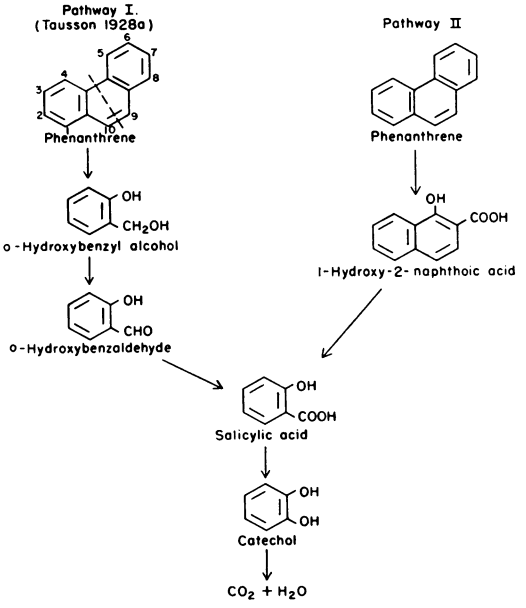

Figure 4. Possible pathways of phenanthrene oxidation.

is given by the residual acid. Decarboxylation could account for the carbon dioxide evolved, and naphthoquinones would give a negative ferric chloride test. Most other possible phenolic intermediates would give positive color reactions with ferric chloride.

The situation may be analogous to that found by Murphy and Stone (1955) for naphthalene dissimilation by a pseudomonad. Apparently two pathways exist for naphthalene dissimilation, one through salicylic acid to carbon dioxide, the other to 1,2-naphthoquinone which concentrated in the medium as an inhibitory product and was not attacked further. The observation in this laboratory that phenanthrene-grown cells have the enzymes necessary to oxidize salicylic acid and catechol also indicates that phenanthrene dissimilation may proceed to carbon dioxide via this known pathway for the oxidation of aromatic rings.

It is interesting to note that phenanthrene is not oxidized by our culture at the 9,10-bond, the most chemically reactive position in the molecule. This is in accordance with the postulate, based on studies of the oxidation of polycyclic compounds in animals (Pullman and Baudet, 1954), that the polycyclics are bound to enzymes at the region of highest electron density by an additional reaction. Oxidation occurs at a nearby bond normally of secondary chemical activity. Attachment of phenanthrene by the 9,10-bond would therefore give primary oxidation at the end ring. However it must be noted that although 1,2-dihydroxy-1,2-dihydrophenanthrene has been isolated from the urine of rabbits injected with phenanthrene, it occurs in a mixture with the 9,10-isomeric diol; when injected into rats only the 9,10-isomeric diol is excreted (Boyland and Wolf, 1950). Phenanthrene oxidation in the animal is somewhat anomalous, since in every other case of polycyclic oxidation in animals that has been studied, attack does not occur at the most chemically reactive position.

The position of 1,2-naphthoquinone in the pathway is of interest. Further experiments are in progress to demonstrate its formation, and whether it lies directly in the oxidative pathway or branches off as a metabolic product whose inhibitory action may enhance the buildup of 1-hydroxy-2-naphthoic acid in the medium.

ACKNOWLEDGMENTS

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SUMMARY

An organism identified as a member of the genus *Pseudomonas* has been isolated which oxidizes phenanthrene as a sole carbon source. Oxidation proceeds via 1-hydroxy-2-naphthoic acid which has been isolated and identified. Further oxidation via salicylic acid and catechol is indicated by simultaneous utilization of these compounds. There are some indications that an alternate pathway to 1,2-naphthoquinone may exist.

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