OXIDATION OF AROMATIC COMPOUNDS BY BACTERIA

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OXIDATION OF AROMATIC COMPOUNDS BY BACTERIA

By

Martin H. Rogoff ² and Irving Wender ³

Summary

THE OXIDATION of polynuclear aromatic hydrocarbons by strains of soil bacteria is discussed in relation to recent findings. Pathways for the degradation of several two- and three-ring compounds are also described, demonstrating that polynuclear aromatic hydrocarbons are degraded by a well-characterized central metabolic path. Intermediary metabolites are mainly hydroxylated aromatic compounds and hydroxyaromatic acids. Salicylic acid, or an analog, is shown as the ultimate product of end-ring fission of polynuclear aromatic compounds by bacteria.

Terminal oxidation of aromatic residues is traced to the aliphatic end products by the compound catechol. These aliphatic products of ring splitting are further degraded by the various constitutive respiratory cycles of the cell.

Observing cross-induction patterns of polycyclic aromatic hydrocarbon-grown soil bacteria and rates at which these bacteria oxidize substituted derivatives of the growth compounds has yielded interesting data. These findings support the hypothesis that polycyclic aromatic hydrocarbons attach to homologous enzymes through regions of high electron density; oxidation and ring cleavage then occur elsewhere on the substrate molecule.

An appendix containing information about some of the techniques used in the study of bacterial oxidation of hydrocarbons is included.

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INTRODUCTION

The structure of coal, a complex organic material, probably consists chiefly of condensed aromatic and hydroaromatic rings. The ring systems in coal resist microbial oxidation; only highly oxidized coals give any indication of supporting an indigenous microflora (14).⁴ Certain polynuclear hydrocarbons, such as phenanthrene, biphenyl, tetralin, acenaphthene, or anthracene, have nuclei that may be structurally related to the "coal molecule." They are also admirable models for determining intermediate products of microbial oxidation of highly condensed ring systems in general.

Coal contains phenolic hydroxyl and is thought to contain quinone and carboxyl groups. Evidence 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, mo-

lecular size, 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.

In recent years metabolic studies, mainly isolating intermediates and determining induced enzyme patterns, have yielded fairly definitive knowledge about the way in which aromatic compounds are metabolized by bacteria. This information is sufficient to give us a wide perspective of the field and to help us visualize certain general metabolic pathways of these aromatic degradations. The material in this paper is about central paths and how bacterial enzymes manipulate and cleave the aromatic ring with the formation of hydroxylated aromatic intermediates. The paper also discusses further cleavage of these hydroxylated compounds to aliphatic intermediates, which at some stage can enter the main terminal respiratory cycles of the bacterial cell.

⁴ Numbers in parentheses refer to items in the bibliography at the end of this report.

OXIDATIVE PATHWAYS

BACTERIAL OXIDATION OF INDIVIDUAL POLYCYCLIC AROMATIC HYDROCARBONS

Oxidation of Naphthalene by Soil Bacteria

Naphthalene, the lowest member of the polycyclic series, is oxidized by soil bacteria through the intermediates shown in figure 1. Strawinski

FIGURE 1.—Degradation of Naphthalene by Soil Bacteria.

and Stone (32), studying the biological detoxification of soil sterilizers, noted the rapid disappearance of naphthalene from soils and isolated pure cultures of bacteria that produced salicylic acid when naphthalene was provided as source of carbon. Determination of the intermediates in the pathway and of other compounds associated with the oxidative process was made as follows.

1,2-DIHYDRO-1,2-DIHYDROXYNAPHTHALENE

Walker and Wiltshire (38) studied a naphthalene-decomposing gram-negative motile bacterium that they isolated from soil by a percolation enrichment technique. The organism was quite similar to *Bacillus naphthalinicus*

nonliquifaciens. A substance was obtained from 48-hour cultures on mineral salts media that yielded 1-naphthol after treatment with dilute hydrochloric acid. Isolation of the naphthol-yielding compound was made by extracting from mass cultures; the resulting compound was identified as a 1,2-dihydro-1,2-dihydroxynaphthalene. Manometric experiments showed that the bacteria grown on naphthalene sequentially oxidized the *D-trans*-diol and the *DL-trans*-diol but not the *L-trans*-diol, thus establishing the *D-trans* form as the actual intermediary metabolite.⁵ The role of the *D-trans*-diol as a metabolite was confirmed later by Fernley and Evans (8).

1,2-DIHYDROXYNAPHTHALENE

Walker and Wiltshire (38) and Murphy and Stone (16) reported that naphthalene-grown resting-cell suspensions of bacteria exhibited small amounts of oxygen uptake in manometric sequential induction experiments when 1,2-dihydroxynaphthalene was a substrate. These investigators were uncertain as to whether the uptake was significant enough to include the compound as an intermediate in naphthalene breakdown. Fernley and Evans (8) demonstrated that bacterial cells of the naphthalene-oxidizing strain were sequentially induced to oxidize 1,2-dihydroxynaphthalene. Crude cell-free extracts of this strain rapidly oxidized the compound; the major product of the enzyme action was coumarin, which was isolated on the acidification and ether extraction from the reaction mixture. 1,2-Dihydroxynaphthalene as an intermediate was thus established.

1,2-NAPHTHOQUINONE

This compound is not an intermediate in naphthalene dissimilation. It was found in culture fluids of naphthalene-oxidizing bacteria by Murphy and Stone (16), who demonstrated that the compound was formed by the autocatalytic oxidation of 1,2-dihydroxynaphthalene under neutral to alkaline conditions.

O-HYDROXY-CIS-CINNAMIC ACID

Successful isolation of this intermediate has not been made. It has, however, been obtained as its dehydration product, coumarin, from crude cell-free extracts metabolizing 1,2-dihydroxynaphthalene (8). The latter is oxidized by liberating 1 mole of carbon dioxide and tak-

⁵ The principle of sequential induction is explained in the appendix.

FIGURE 2.—Probable Reactions Following Ring Splitting of 1,2-Dihydroxynaphthalene.

ing up 3 atoms of oxygen per mole of substrate. Analogy with previous work in this field (6) led Fernley and Evans to postulate that *o*-carboxy-*cis*-cinnamic acid is the immediate product of ring splitting. Production of *o*-hydroxy-*cis*-cinnamic acid could result from an oxidative decarboxylation of the labile precursor, and the molar gas exchange data support this supposition. These steps are shown in figure 2.

COUMARIN

Isolation of coumarin from 1,2-dihydroxynaphthalene oxidation does not place this compound as an intermediate because oxygen uptake rates are quite slow when coumarin is used as a substrate for sequentially induced cells. Its role is probably that proposed by Fernley and Evans (8) as shown in figure 2. Additional evidence for nonparticipation of coumarin in the direct pathway was that it was isolated from the acid-ether fraction of the culture fluid; normally it would be obtained from the neutral fraction, whereas *o*-hydroxy-*cis*-cinnamic acid would normally be obtained from the acid fraction.

SALICYLIC ACID

Strawinski and Stone (32), Walker and Wiltshire (38), Murphy and Stone (16), and Fernley and Evans (8) reported formation of salicylic acid in cultures of bacteria oxidizing naphthalene. The latter three papers described procedures establishing the compound as an intermediate by sequential induction. The compound is actually a key intermediate, and it, or an analog, is the ultimate product of end-ring fission of polycyclic hydrocarbons by bacteria. Salicylic acid is metabolized via catechol, described in the appendix.

MELILOTIC ACID AND O-HYDROXY-TRANS-CINNAMIC ACID

Fernley and Evans (8) showed that cultures utilizing naphthalene contained detectable

FIGURE 3.—Degradation of 2-Methylnaphthalene by Soil Bacteria.

amounts of these two compounds. Sequential induction experiments did not successfully identify them as intermediates. They can rise from known biochemical reactions (isomerization, reduction) on the intermediate *o*-hydroxy-*cis*-cinnamic acid and may be metabolized further through as yet unknown metabolic routes.

Dissimilation of Methylnaphthalenes

Strawinski observed that the methylnaphthalenes were less readily available to soil bacteria than the parent compound, naphthalene (31). Oxidation of 1- and 2-methylnaphthalene parallels the pathway for naphthalene dissimilation with the intermediary metabolites bearing the methyl substituent (fig. 3); the methyl group is apparently not lost until after cleavage of the final aromatic residue. Another interesting feature is the existence of an alternate oxidative pathway indicated by recovery of 2-naphthoic acid from still cultures of a soil pseudomonad oxidizing 2-methylnaphthalene (24). Following is a description of the salient features of the 2-methylnaphthalene degradation pathway.

7-METHYL-1,2-DIHYDRO-1,2-DIHYDROXY-NAPHTHALENE

The methyl-substituted dihydrodiol [(+)-*trans*] was isolated by Canonica and his associates (1) from culture fluids of a strain of *Pseudomonas* having 2-methylnaphthalene as

a source of carbon. The material was recovered by ether extraction of the medium, following filtration to remove the alkaline precipitate, and then was recrystallized from heptane. This finding suggests that the same mechanism of ring splitting found in naphthalene is in operation.

7-METHYL-1-NAPHTHOL

This compound is not an intermediate in the degradative pathway. However, it was obtained by Canonica and associates (1) as a dehydration product on acidification of culture fluids containing the dihydrodiol. (See preceding paragraph.)

2-HYDROXY-4-METHYLBENZOIC ACID

Canonica and associates (1) reported isolation of this intermediate from the same culture fluids which contained the dihydrodiol, previously mentioned. Treccani and Fiecchi (36) applied the sequential induction technique to the compound and noted that the organism oxidized the compound but not the 3-methyl isomer; 2-hydroxy-3-methylbenzoic acid was isolated by Rogoff and Wender (24) from 1-methylnaphthalene degradation by a soil pseudomonad. These sequential induction experiments by Rogoff and Wender showed that the methylsalicylic acids and their respective methylcatechols were intermediates in the methylnaphthalene oxidations. Intermediates with the methyl group *ortho* to the hydroxyl were, however, oxidized more slowly than those with the methyl group in the *meta* or *para* position. The effect, apparently steric, was strongest in 3-methylsalicylic acid, a possible explanation of Treccani and Fiecchi's observation. A significant part of the studies was that cells that were grown on naphthalene, or either of the methylnaphthalenes, were induced to oxidize salicylic acid and catechol and their methyl derivatives. This suggests that the same enzymes elaborated for oxidizing the parent compounds (salicylic acid, catechol) are involved in oxidizing the methyl derivatives; the presence of the methyl group on the benzene nucleus apparently does not confer a specificity requirement on the enzymes.

4-METHYLCATECHOL

This compound has not been isolated from 2-methylnaphthalene-oxidizing cultures, yet it obeys the criteria of sequential induction (24). By analogy to other pathways, it is not unlikely that the compound is a true intermediate and a product of the oxidative decarboxylation of 2-hydroxy-4-methylbenzoic acid.

2-NAPHTHOIC ACID

When cultures oxidizing 2-methylnaphthalene were not subjected to forced aeration (shaking), Rogoff and Wender (24) isolated a crystalline material by ether extraction that was identified as 2-naphthoic acid. 2-Methylnaphthalene-grown cells were, however, not induced to oxidize the compound, nor were those grown on 1-methylnaphthalene induced to oxidize 1-naphthoic acid. With present evidence it seems premature to classify the compounds as metabolic end products, even though they may be. However, purely chemical oxidation of the methyl to a carboxyl group is unlikely. If a chemical factor is involved in production of naphthoic acids, a group in a higher state of oxidation than the methyl is more likely to be oxidized. At present the only point that can be made about 2-naphthoic acid production is that an alternative oxidative pathway is indicated.

The two pathways do not seem to be mutually exclusive; infrared spectra of the material from certain culture fluids indicated the presence of both 2-hydroxy-4-methylbenzoic acid and 2-naphthoic acid. It is entirely possible that one pathway will dominate under any given set of conditions—the bulk of the cells possessing the enzymes necessary for the given set of reactions.

Oxidation of Phenanthrene and Anthracene

Several of the intermediates in the degradation of these two compounds were identified independently in two laboratories, and the pathways seem quite similar, therefore, they will be discussed together. Oxidation of phenanthrene by soil bacteria was observed in 1928 by Tausson (35). Based on the rates at which his organism utilized certain postulated intermediates, Tausson postulated a microbial attack on the chemically reactive 9, 10-bond. We now know that this is not so, and end-ring attack seems the general mode of microbial oxidation of polynuclear aromatic hydrocarbons. What is known about the intermediates in phenanthrene and anthracene degradation by soil bacteria is summarized in figure 4.

3,4 - DIHYDRO - 3,4 - DIHYDROXYPHENANTHRENE AND 1,2-DIHYDRO-1,2-DIHYDROXYANTHRACENE

Colla and coworkers (3) studied phenanthrene and anthracene oxidation by a species of *Flavobacterium*. In cultures run in 20-liter fermentors they found, after short incubation periods, evidence of materials giving a positive test for phenols with the reagent 2,6-dichloro-

FIGURE 4.—Dissimilation of Phenanthrene and Anthracene by Soil Bacteria.

quinonechlorimide after treatment with hydrochloric acid. Suitable extraction of the medium yielded a compound identified as 3,4-dihydro-3,4-dihydroxyphenanthrene by elemental analysis, paper chromatography, and identifying derivatives. Dehydration products of the compound, 3- and 4-phenanthrol, were also identified.

The anthracene diol has not yet been isolated, even though positive reactions denoting phenol in the culture media were observed; the 1,2-anthracene diol has been postulated as the probable intermediate.

1-HYDROXY-2-NAPHTHOIC ACID AND 2-HYDROXY-3-NAPHTHOIC ACID

Isolation of these products from the attack on phenanthrene and anthracene, respectively, by species of *Pseudomonas* was reported by Rogoff and Wender (22, 23); Colla and coworkers independently made the same observation with a species of *Flavobacterium* (2). Isolating these compounds demonstrated that attack on phenanthrene and anthracene was directed toward an end ring and not toward the chemically reactive carbons of the center ring. It should be noted that the hydroxynaphthoic acids are the next higher homologs of salicylic acid in the aromatic series and that apparently the mechanisms of attack on phenanthrene and anthracene parallel that on naphthalene.

SALICYLIC ACID

Rogoff and Wender (22, 23) tested suspected intermediates as substrates for enzymes induced in their pseudomonad when they were grown with phenanthrene or anthracene as source of carbon. Salicylic acid and catechol fulfilled the criteria of sequential induction and were proposed as intermediates. Later, Colla and associates (3) succeeded in isolating small amounts of salicylic acid from phenanthrene culture fluids by sublimation of the materials obtained from acid-ether extracts. Catechol has not been isolated from hydrocarbon-oxidizing cultures.

MODE OF OXIDATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY BACTERIA

General Mode of Oxidation

From the preceding discussion it can be seen that bacterial oxidation of aromatic compounds, in general, proceeds through a series of intermediary "type" compounds. When the substrate is polynuclear, reactions equivalent to the addition of 1 mole of H_2O_2 occur with the formation of a dihydroaromatic diol. This type dehydrogenates to a compound having two hydroxyl groups in the *ortho*-configuration, and the ring is cleaved between them. The salicylic acid "type" intermediate is the compound preceding catechol; its carboxyl group represents the residuum of prior ring splitting, or side-chain oxidation. This general mode of ring splitting is summarized in figure 5.

FIGURE 5.—General Mode of Ring Splitting (Schematic).

Terminal Oxidation

The final product of end ring-fission of polycyclic aromatic compounds is salicylic acid, or an analog. There are exceptions, but this compound represents the main metabolic path to carbon dioxide and water. Salicylic acid undergoes direct oxidative decarboxylation to form catechol (25). Catechol is in turn cleaved between its *o*-hydroxyl groups to form aliphatic intermediates which can enter the terminal respiratory cycles. The efforts of several workers have elucidated the steps involved in degradation of catechol; these are shown in figure 6.

FIGURE 6.—Oxidative Metabolic Pathway of Catechol by Microorganisms.

Catechol is split between the hydroxyl groups to form *cis-cis*-muconic acid. Tausson (35) had suggested that the benzene nucleus was split to form muconic acid, by phenanthrene-oxidizing bacteria, and then oxidized to carbon dioxide. Evans and Happold (6) excluded *trans-trans*-muconic acid as a possible intermediate on the basis of the inability of their phenol- and catechol-oxidizing *Vibrio* 0/1 to grow on or to metabolize the compound. Hayaishi and Hashimoto (9) tentatively identified as *cis-cis*-muconic acid a product of the oxidation of catechol by a purified *Pseudomonas* enzyme. Stanier and Hayaishi (29) used the acid obtained by Hayaishi and Hashimoto as a substrate for a crude enzyme preparation that catalyzed the oxidation of catechol to β -oxoadipic acid. The preparation did not yield the oxo-acid from the muconic acid, excluding it as a true intermediate. It had been shown by Elvidge and associates (5) that a *cis-trans*-muconic acid they had prepared chemically was virtually indistinguishable from the *cis-cis*-muconic acid; simply boiling the *cis-cis*-acid inverted it to the *cis-trans* form. Apparently the treatment used by Stanier and Hayaishi to purify the *cis-cis* acid in their microbiological studies inverted the samples. The picture was clarified by the work of Evans (6) who showed that the pure *cis-cis*-isomer was metabolized by intact cells, and was nonoxidatively converted into β -oxoadipic acid by a crude catechol-oxidizing cell-free preparation; the *cis-trans*- and *trans-trans*-isomers were inactive.

It was found by Evans' group (7) that the lactone γ -carboxymethyl- Δ^{α} butenolide, related to *cis-cis*-muconic acid, could be converted to β -oxoadipic acid by the same cell-free enzyme

preparation. Converting *cis-cis*-muconic acid to β -oxoadipic acid is done by a lactonizing enzyme and a lactone-splitting enzyme; these have been separated by Sistrom and Stanier (26, 27). The lactonizing enzyme, which requires Mg^{++} or Mn^{++} ions for activity, catalyzes the reversible interconversion of *cis-cis*-muconic acid and the (+)-lactone. This enzyme can also convert the *cis-trans*-isomer into an equilibrium mixture with the (−)-lactone, but more slowly than with the natural substrate.

Evans, studying phenol breakdown by a *Vibrio*, found a compound that appeared transiently in the medium and that gave positive Rothera and Gerhardt reactions characteristic of a β -oxo-acid (6). The compound was isolated by Kilby (13) and identified as β -oxoadipic acid. Thus the steps in the degradation between *cis-cis*-muconic acid and β -oxoadipic acid are complete—the last compound in the chain, however, being the first isolated and identified. The final step before entry of the compounds into the terminal respiratory cycles was shown by Kilby (13) to be a $C_{(4)}-C_{(2)}$ split of β -oxoadipic acid to succinate and acetate. The enzymology of the C_4-C_2 split was worked out by Katagiri and Hayaishi (12). They prepared a cell-free enzyme from a *Pseudomonas* *sp.* which metabolized β -oxoadipic acid in the presence of coenzyme A (CoA) and succinyl coenzyme A (SuccCoA). In the primary reaction catalyzed by a specific thiophorase, SuccCoA acts as a CoA donor and β -oxoadipyl-CoA is produced. In a second reaction, β -oxoadipylCoA is cleaved in the presence of a specific thiolase to yield SuccCoA and acetyl-CoA. The SuccCoA is utilized again as a CoA donor.

Directing Influence of Electron-Rich Bonds in Aromatic Substrates on the Site of Bacterial Attack

Manometric experiments with polycyclic aromatic hydrocarbon-grown cells of the strains of soil bacteria under study in the Bureau of Mines laboratory have yielded considerable data as to the induced enzyme patterns of these bacteria; a summary is given in tables 1 and 2. The salient features of these data are:

1. Substitution of phenanthrene by a methyl group at the 9-carbon blocks of oxidation of the compound by the phenanthrene- (or other) grown organism.
2. Oxidation of 2-methylphenanthrene is considerably higher while 3-methylphenanthrene is oxidized at a rate intermediate between the rates of the 9- and 2-isomers. The 3-carbon is located on a ring-splitting site.
3. Naphthalene- or anthracene-grown cells oxidize phenanthrene at lower rates than cells grown with phenanthrene or 2-methylnaphthalene as the source

TABLE 1.—*Respiration of polycyclic aromatic hydrocarbon-grown pseudomonads on hydrocarbon substrates*

Cells grown on—	Substrate	Q _{O₂} (N) ¹	Relative oxidation rate (naphthalene=1.00)
<i>Cross-induction studies</i>			
Naphthalene.....	Naphthalene.....	672	1.00
	2-Methylnaphthalene.....	456	.73
	Phenanthrene.....	69	.11
Anthracene.....	Naphthalene.....	305	1.00
	2-Methylnaphthalene.....	157	.52
	Phenanthrene.....	37	.12
Phenanthrene.....	Naphthalene.....	374	1.00
	2-Methylnaphthalene.....	359	.96
	Phenanthrene.....	110	.30
2-Methylnaphthalene.....	Naphthalene.....	639	1.00
	2-Methylnaphthalene.....	618	.97
	Phenanthrene.....	359	.56
<i>Substituted naphthalenes</i>			
Naphthalene.....	Naphthalene.....	720	1.00
	2,3-Dimethylnaphthalene.....	214	.30
	2,7-Dimethylnaphthalene.....	67	.09
Phenanthrene.....	Naphthalene.....	956	1.00
	2,3-Dimethylnaphthalene.....	310	.32
	2,7-Dimethylnaphthalene.....	464	.49
2-Methylnaphthalene.....	Naphthalene.....	742	1.00
	2,3-Dimethylnaphthalene.....	312	.42
	2,7-Dimethylnaphthalene.....	378	.51

¹ Q_{O₂}(N) represents microliters of O₂ taken up per milligram of cell N per hour.

TABLE 2.—*Respiration of soil pseudomonads grown on different polynuclear aromatic compounds in the presence of phenanthrene and some monomethyl derivatives*¹

Substrate	Q _{O₂} (N) ² of cells grown on—		
	Naphthalene	Phenanthrene	2-Methylnaphthalene
Phenanthrene.....	172	716	472
2-Methylphenanthrene.....	128	396	230
3-Methylphenanthrene.....	74	210	188
9-Methylphenanthrene.....	28	78	42

¹ Oxygen uptake was determined in Warburg manometers with 10 micromoles of substrate provided. Endogenous respiration was subtracted before calculation.

² Q_{O₂}(N) represents microliters of O₂ taken up per milligram of cell N per hour.

of carbon. In general, cells grown on 2-methylnaphthalene display induced enzyme patterns closely resembling the phenanthrene-grown cells, while those grown on anthracene and naphthalene are related in the patterns they display.

4. Naphthalene-grown cells oxidize 2,3-dimethylnaphthalene at a higher rate than they oxidize the 2,7-dimethylnaphthalene. The reverse is true if the cells are grown on phenanthrene or 2-methylnaphthalene.

The phenomena observed may be related to the presence on polycyclic aromatic hydrocarbons of regions of high electron density termed by Pullman and Pullman (21) the K-region, as exemplified by the 9- and 10-carbons of phenanthrene, and the L-region, as found at the 9- and 10-carbons of anthracene.

Any attempt to explain observed phenomena must be based on an understanding of the chem-

ical characteristics of molecular structures undergoing oxidation by these bacteria. Not all the bonds in polynuclear aromatic compounds are equivalent. For example, many observations indicate great differences between the properties of the 1,2-bond and the 2,3-bond of naphthalene (4). In addition reactions, with which we are concerned, starting the process of adding to an aromatic bond seems to be converting it into a localized double bond. In the process, two π -electrons, of the general π -electron system of the compounds, are fixed at the localization site; remaining electrons may re-group to form a minimum-energy system. The energy necessary to localize double bonds in many aromatic compounds has been calculated by the molecular-orbital method (4). The localization energy for the 1,2-bond in naphthalene has been calculated to be 22 kcal.; that for the 2,3-bond is 37 kcal. while that for the 9,10-bond in phenanthrene is only 16 kcal. A value of 30 kcal. has been calculated for a bond in benzene. The low localization energies for the 1,2-bond in naphthalene and the 9,10-bond in phenanthrene explain the reactivity of those bonds in addition reactions. The bond in an aromatic molecule having the lowest localization energy requirement may be considered that bond which Pullman and Pullman (21) call the K-region.

Participation of the 9,10-bond of phenanthrene in oxidizing the compound by the Bureau culture (table 2) and recognition of that bond as the site of addition reactions lead us to postulate attachment of a phenanthrene

molecule to an enzyme by an addition reaction with the 9- and 10-carbons. Adding to an enzyme should occur in the case of naphthalene and anthracene at a 1,2-bond. With 2-methylnaphthalene, the presence of the methyl group on one of the K-region carbons should cause increase of the electron density at that bond, also introducing steric effects on addition reactions (19). It therefore cannot be stated with any certainty at which bond the addition to an enzyme might occur, but it is apparent that the induced enzyme patterns observed for 2-methylnaphthalene-grown cells are quite similar to those of their phenanthrene-grown counterparts.

An aromatic compound with electronic characteristics that directs addition to an enzyme at a particular bond imposes limitations on that enzyme as to which other bonds in the molecule will act as a site for its oxidative (or other) function. This specificity may be due to activation in the enzyme-substrate complex of a bond of normally secondary reactivity in the substrate molecule (20). Specificity may also be imposed sterically owing to configurations of the molecules. Thus, attack on naphthalene may be on the 3,4-bond following addition to an enzyme on the 1,2-bond on the same ring. This is supported by the very low rate of oxidation by naphthalene-grown cells of 2,7-dimethylnaphthalene (table 1) in contrast to the rate of oxidation of the 2,3-isomer. Such cells also oxidize phenanthrene at a low rate (table 1); in this case addition to an enzyme and oxidation probably occur on different rings. Phenanthrene-grown cells, however, oxidize the 2,7-dimethylnaphthalene more readily than the 2,3-isomer, the former compound having one unblocked bond in each of its rings. In accord with the postulate, anthracene oxidation should result in a naphthalene-type induced enzyme pattern (K-region at the 1,2-bond), and this has been observed (table 1).

Additional support for these postulates is given when absorption of polynuclear aromatic hydrocarbons from aqueous solutions by suspensions of hydrocarbon-grown cells is measured. Because the molecular size of the test compounds might prevent their entry into the cell, it was felt that their oxidation occurred on the cell wall; the absorptive capacity of the cell wall might, therefore, reflect the activities of the enzymes it contained. Ultraviolet spectra of samples of polynuclear aromatic compounds were determined, and their solubilities were calculated (figure 7). The procedure used to determine absorption of the hydrocarbons from solution by suspensions of resting cells follows:

FIGURE 7.—Ultraviolet Spectra of Polycyclic Compounds in M/25 Phosphate Buffer, pH 7.38.

40-ml. aliquots of the filtered hydrocarbon solutions were placed in 75-ml. Lusteroid⁶ centrifuge tubes. Cell suspensions were adjusted to contain 1 mg. bacterial N per milliliter, and 1-ml. aliquots of various dilutions were added to the tubes; a blank tube received 1-ml. of buffer. The tubes were allowed to stand for 3 minutes and then centrifuged at 3,500 times gravity for 20 minutes. The supernatant fluids were decanted, aliquots were placed in the ultraviolet cells, and the spectra were recorded. When phenanthrene absorption was measured, a 10-cm. quartz cell was used; the 1-cm. cell was adequate for the more soluble naphthalene. Concentrations of the hydrocarbons were calculated from the spectra by the baseline method (39). Data obtained from several experiments are summarized in table 3, representing the average values of at least two dilutions of the same cell suspension. It is readily seen that the naphthalene-grown cells do not absorb phenanthrene from solution as much as do the phenanthrene-grown suspensions, although the amount of naphthalene absorbed does not differ significantly.

⁶ Reference to specific brands is made to facilitate understanding and does not imply endorsement of such items by the Bureau of Mines.

TABLE 3.—*Absorption of naphthalene and phenanthrene from aqueous solution by resting cells of a soil pseudomonad grown with one of the compounds as a source of carbon*

Cells grown on—	Hydrocarbon	Solubility milligrams per liter	Hydrocarbon absorbed per milligram of bacterial N		Absorption ratio, naph- thalene to phenanthrene
			milligrams	Moles $\times 10^{-6}$	
Naphthalene.....	Naphthalene.....	37.0	0.279	2.18	3.6
	Phenanthrene.....	1.5	.078	.44	
Phenanthrene.....	Naphthalene.....		.388	3.03	1.1
	Phenanthrene.....		.485	2.72	

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⁷ Titles enclosed in brackets are translations from the language in which the item was published.

APPENDIX.—METHODS FOR STUDY OF BACTERIAL OXIDATION FOR AROMATIC COMPOUNDS

This appendix describes the general bacteriological methods used in studies of microbial hydrocarbon degradation, which should be of interest to those engaged in or considering study in this field.

is used to inoculate flasks of mineral salt media containing the hydrocarbon under study. Routine bacteriological isolation techniques are then used to obtain pure cultures of desired bacteria (15).¹

ORGANISMS

Over 100 species of microorganisms have been reported able to utilize hydrocarbons as sources of carbon and energy; however, of these, the number capable of utilizing aromatic hydrocarbons is relatively small. Examples of bacteria that oxidize aromatic hydrocarbons are given in table A-1. Of these, the members of the genus *Pseudomonas* and the closely allied *Flavobacteria* seem, by far, the most active. It is difficult to determine which of the *Bacillus* and *Bacterium* designations in table A-1 actually represent species that belong to the genus *Pseudomonas*. Undoubtedly, several do. The source of these organisms is usually soil, which

MEDIA

In the light of present knowledge, microbial attack on the aromatic ring seems to be dependent on induced enzyme formation and activity. Since the presence of other carbonaceous material in the medium (more available to the organism due to oxidizability by constitutive enzymes) might inhibit induced enzyme formation, growth in a purely mineral salts medium becomes the method of choice. In general, a suitable medium has a mineral content balanced to meet the osmotic and nutritional requirement of the organism—a source of nitrogen (nitrate

¹ Italicized numbers in parentheses refer to items in the list of references preceding this appendix.

TABLE A-1.—*Bacteria that utilize aromatic hydrocarbons*

Genus and species	Hydrocarbons utilized	Investigators
<i>Pseudomonas aeruginosa</i>	Naphthalene..... Phenanthrene..... Methylnaphthalene.....	Strawinski and Stone (32). Rogoff and Wender (23). Rogoff and Wender (24).
<i>Ps. boreopolis</i>	Naphthalene.....	Murphy and Stone (16).
<i>Bacterium benzoli</i>	Benzene, toluene, xylene.....	Norris (17).
<i>B. naphthalenicus</i>	Naphthalene.....	Tattersfield (33).
<i>B. phenanthrenicus</i>	Phenanthrene.....	Do.
<i>Bacillus hexacarbovorum</i>	Benzene, xylene, toluene.....	Stormer (30).
<i>B. toluolicum</i>	do.....	Tausson (34).
<i>Flavobacterium spp.</i>	Phenanthrene, anthracene.....	Colla, Biaggi, and Treccani (2).

TABLE A-2.—*Composition of media used in studies of bacterial oxidation of hydrocarbons, grams per liter*

Organism	Investigator	MnCl ₂	Mg(NH ₄)PO ₄	MgSO ₄	K ₂ HPO ₄	Na ₂ HPO ₄	KH ₂ PO ₄	NH ₄ NO ₃	NH ₄ Cl	CaSO ₄	CsCO ₂	CaCl ₂	NaCl	FeCl ₃	Traces
<i>Bacterium aliphaticum</i>	Johnson and Schwartz (11).	-----	1.0	-----	0.8	-----	-----	-----	-----	0.1	-----	-----	-----	(¹)	K, I
<i>Methanomonas spp.</i>	Sohnngen (28)	-----	1.0	-----	.5	-----	-----	-----	-----	.1	-----	-----	-----	-----	-----
<i>Pseudomonas aeruginosa</i>	Imelik (10)	-----	-----	0.5	-----	1.0	0.5	-----	0.5	-----	-----	-----	4.0	0.05	Mn, K
<i>Pseudomonas spp.</i>	Strawinski (31)	-----	-----	.5	-----	1.0	.5	2.5	-----	-----	2.0	-----	-----	-----	-----
<i>Pseudomonas spp.</i>	Stanire and Hayaishi (29)	-----	-----	.5	1.0	-----	-----	1.0	-----	-----	-----	-----	-----	-----	-----
<i>Pseudomonas spp.</i>	Rogoff and Wender (24) ²	0.1	-----	.5	-----	1.0	.5	2.5	-----	-----	2.0	0.01	-----	.05	-----

¹ Trace.

² Yeast extract 0.05 percent was used as a vitamin supplement.

or ammonium ion) and a pH near neutrality. An organic source of nitrogen may prove stimulatory, but care must be taken to maintain low enough levels so that the organic carbon of the nitrogen source does not provide a competitive source of carbon and/or energy. Phosphate is necessary in most media to obtain optimum growth levels. It has been learned from experience that additives such as yeast extract or digests of casein are beneficial as long as their concentration is sufficiently low (less than 0.1 percent). Some of the media used by various investigators in hydrocarbon oxidation studies are listed in table A-2.

CULTURE CONDITIONS

Substrates

Since most of the hydrocarbons studied are insoluble in water, and the rate of their attack depends on surface area available for contact between substrate and bacterium, dispersion of the substrate in the medium becomes a problem. In most cases grinding the material finely is sufficient treatment because by vigorous agitation and growth of the bacteria the hydrocarbon becomes wetted and intimately admixed in the flask medium. This may not be done with liquid hydrocarbons that tend to agglomerate. Oxidation of these is enhanced if some inert material such as sterile agar or kaolite is used as an adsorption agent on which the hydrocarbon is placed before adding the medium. We have also had some success adding a small amount of a surfactant (Tween 80, Dow Aerosol 18) to the medium as a dispersion aid.

Growth on agar media with such substrates as naphthalene or methylnaphthalene, which have relatively low boiling points, is easily accomplished by inverting containers of solid media over the substrate. The bacteria grow readily over the vapors. Growth on agar media with compounds having low vapor pressures can be enhanced if the finely dispersed hydrocarbon is sprinkled directly onto the surface of the agar, and a small amount of sterile medium is added, sufficient to maintain a moist surface.

Inoculum

The rule of thumb is that 5 to 10 percent is a minimum inoculum in experiments in liquid media. The media used, unless containing a vitamin supplement, are probably insufficient to meet the nutritional requirements of the bacteria. This can be overcome by a large inoculum that would contain lysed cells and

undetermined "leakage factors," as well as an initial source of organic nitrogen. The large inoculum has the additional advantage of decreasing the time lag for obtaining a maximum bacterial population, useful when the isolation of intermediate compounds is desired.

Temperature

Optimal temperatures of incubation vary with the organism under study; for most soil forms the range should be 20° to 30° C. (18).

Aeration (Oxygen Tension)

The bacterial attack on hydrocarbons is essentially oxidative; thus oxygen availability is a prime factor. In general, the rate of attack on a hydrocarbon will bear a direct relationship to the amount of oxygen available (within limits). The effects of oxygen may be even more profound. Thus, Rogoff and Wender (24) found that aerated (shaken-flask) 2-methylnaphthalene cultures yielded 2-hydroxy-4-methylbenzoic acid as an intermediate, but unshaken, shallow-layer cultures yielded an additional product from an alternate path, 2-naphthoic acid. Therefore, rapid oxidation may not yield the most information. Aeration may be achieved by shaking, stirring, percolation, or direct introduction of sterile air by bubbling it through the medium. The method of aeration used will depend on the problem being studied.

CRITERIA FOR ESTABLISHMENT OF INTERMEDIATES

Isolation of Pure Compounds

Bacteria usually degrade the hydrocarbon substrates to carbon dioxide and water, but at some point in the growth of the culture they may accumulate an intermediate compound in the medium. These may be isolated chemically from the culture fluids. An example of such procedure is the method used (22) for recovery of 1-hydroxy-2-naphthoic acid from phenanthrene fermentation. Cultures were pooled and concentrated under reduced pressure to approximately 50 percent of their volume. The concentrate was acidified with 6*N* hydrochloric acid and was continuously extracted with ether for 24 hours. The ether was extracted with 10 percent sodium bicarbonate, and the bicarbonate layer was separated and acidified with concentrated hydrochloric acid, giving a white precipi-

tate. Ether was added, and the mixture was 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 were dried with anhydrous sodium sulfate. The ether was evaporated under a stream of nitrogen, giving 0.84 gram of a crystalline material. Recrystallization from benzene-acetic acid yielded 0.46 gram of white crystals, melting point 196.5° to 197° C. The infrared and ultraviolet spectra were identical with those of an authentic sample of 1-hydroxy-2-naphthoic acid. Designating isolated compounds as intermediates is substantiated by use of the isolated compound by the organism; that is, it should fit the criteria of sequential induction, described next.

Sequential Induction Technique

The enzymes that oxidize and otherwise manipulate hydrocarbon substrates are usually produced in specific response to the presence of the homologous substrate in the culture fluid. These are called induced enzymes in contrast to constitutive enzymes of the cell that are always formed by a given species regardless of presence or absence of a particular substrate in the medium. The sequential induction technique is best illustrated by a hypothetical metabolic pathway.

for degradation of compound *A*. Cells grown in the absence of compound *A* will not contain the enzymes relevant to the pathway. If grown in the presence of *A*, the cells will produce enzyme *Ea*, catalyzing the step *A*→*B*. The

presence of *B* will induce formation of enzyme *Eb* and so on down the line of intermediates; cells that oxidize *A* completely must also contain the enzymes necessary to oxidize *A* through *E*, and so on. If compound *C* is used as the inducing compound, then the cells should contain the enzymes necessary to oxidize *C*, *D*, *E*, and so forth, but not necessarily *A* and *B*. If we isolate suspected intermediate compound *U*, and cells adapted to compound *A* do not oxidize compound *U*, or do so only after a lag period, it is quite unlikely that compound *U* is a member of the reaction series. The induction response is highly specific. Uninduced cells must be used as a check to insure that the enzymes involved are indeed of the induced type.

The Warburg apparatus is frequently used in these studies with oxygen uptake rates as measures of oxidative activity (37). Washed cell suspensions grown on the hydrocarbon are incubated separately with various suspected intermediates as substrates. The absence of a lag period means that the compound meets the criteria of sequential induction. Results are not unequivocal. If a cell is not freely permeable to the compound treated, very small lag periods may occur that must be interpreted as noninduction. Also, specificity of the response must not be doubted. Supported by other evidence, this method is the best available. It is one of the most useful techniques available for studying degradation of aromatic compounds.

Specific Enzymes

Most desirable and seldom achieved due to lability, particulate nature, and so forth, is the isolation of individual enzymes catalyzing each step in the reaction chain. These provide useful information about cofactor requirements, nonaccumulating intermediates, and mechanisms. Some elegant studies of this type have been made by Sistrom and Stanier (26, 27).

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