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Microbial Degradation of Aromatic Compounds

Metabolic pathways reveal a general formula for the degradation of several aromatic compounds.

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Many synthetic chemicals are added to our environment in the form of herbicides, pesticides, and industrial effluents, and many of these are derivatives of benzene. If such chemicals prove recalcitrant to microbial decomposition they could accumulate in the soil. Such an accumulation could lead to serious ecological changes. Also, there is a possibility that eventually these compounds will localize in animal tissues. In order to prevent such an occurrence, it is vital that we understand how microorganisms degrade both natural and synthetic chemicals.

The aromatic nucleus has a large negative resonance energy; consequently, benzene and its derivatives form a stable group of organic compounds. In spite of the stability of the aromatic nucleus, there are microorganisms which, under the mildest conditions, can completely degrade the benzene ring.

Interest in the microbial degradation of aromatic compounds centers on (i) the study of the metabolic intermediates involved in the degradation of different aromatic substrates, (ii) the elucidation of the enzymatic mechanisms of hydroxylation and ring fission, and (iii) the relatively recent investigations into the control of the enzymes taking part in the metabolism of aromatic compounds. Several reviews are available on the first two areas (*1–4*). In this paper, only recent advances in the microbial degradation of aromatic compounds are discussed.

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Preparation for Ring Fission

It is generally accepted that dihydroxylation is a prerequisite for enzymatic fission of the benzene ring. The hydroxyl groups may be *ortho* to each other, as in catechol and protocatechuic acid, or *para* to each other as in gentisic and homogentisic acids. Catechol and protocatechuic acid have been shown to be the substrates for ring fission in the microbial degradation of many different aromatic compounds (Table 1). Although catechol is an intermediate during the degradation of polynuclear aromatic hydrocarbons—napththalene, anthracene, and phenanthrene—by microorganisms, a dihydroxylated polyaromatic compound is usually the first substrate for ring fission.

A substituted aromatic nucleus presents microorganisms with a choice as to their mode of attack. Results obtained in studies of the microbial degradation of phenyl-substituted acids by a *Nocardia* sp. indicate that the acid side chain is metabolized by a beta-oxidation process; that is, the microorganism removes two carbon atoms at a time (*5*). Thus, side chains with an odd number of carbon atoms are metabolized to benzoic acid, which is converted to catechol prior to ring fission. Phenyl-substituted acids that contain an even number of carbon atoms are metabolized through phenylacetic acid. The further metabolism of phenylacetic acid may proceed through either homogentisic acid (2,5-dihydroxyphenylacetic acid) (*6*) or homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid) (*7*), depending on the species of microorganism.

Studies (*8*) on the metabolism of toluene and isopropylbenzene by *Pseudomonas putida* have shown that these compounds are converted to *ortho*-dihydroxy compounds in which the side chain is left intact. The results of these observations add to a growing list of findings that many aromatic compounds undergo enzymatic hydroxylation of the aromatic nucleus in preference to degradation of the aliphatic side chain. Perhaps the most impressive substrate for ring fission, in terms of the size of its substituent, is 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, an intermediate in the degradation of androsta-4-ene-3,17-dione by *Nocardia restrictus* (*9*). Several examples of substituted aromatic compounds and their respective ring-fission substrates are illustrated in Table 2. Although the compounds that undergo ring fission may vary in their nuclear substitutents, they all have two hydroxyl groups.

The enzymes catalyzing the hydroxylation of the aromatic ring have been termed mixed-function oxidases (*10*). In such reactions one atom of oxygen is incorporated into the substrate molecule. In the presence of a suitable electron donor the other atom of the oxygen molecule is reduced to water. A typical reaction may be represented by the equation,

$$R\text{-}H + O_2 + XH_2 \rightarrow ROH + H_2O + X$$

where R-H represents the substrate molecule and XH_2 represents the electron donor.

Mixed-function oxidation is not peculiar to the metabolism of aromatic compounds, and the importance of such reactions in biological oxygen fixation cannot be overemphasized.

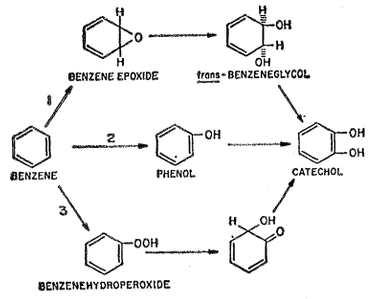


Fig. 1. Hypothetical pathways for the formation of catechol from benzene.

Microorganisms apparently utilize a variety of electron-donating compounds in hydroxylation reactions. Salicylate hydroxylase, the enzyme that converts salicylic acid to catechol, has been purified from cells of a *Pseudomonas* sp. (*11*). The enzyme contains 1 mole of flavin adenine dinucleotide (FAD) per mole of enzyme protein. Since the reaction also requires reduced nicotinamide adenine dinucleotide ($NADH_2$) and oxygen, the mechanism is pictured as a reduction of an enzyme-FAD-salicylate complex by $NADH_2$. Subsequent oxygen fixation by the complex results in the liberation of carbon dioxide with the formation of catechol and the oxidized enzyme.

The enzyme that forms protocatechuic acid from *p*-hydroxybenzoic acid also contains 1 mole of FAD per mole of enzyme protein (*12*). In contrast to salicylate hydroxylase, this enzyme requires reduced nicotinamide adenine-dinucleotide phosphate ($NADPH_2$) for activity.

Phenylalanine hydroxylation is catalyzed by an enzyme present in cells of a *Pseudomonas* sp. Enzymatic activity is dependent on the presence of dimethyltetrahydropteridine and $NADH_2$ (*13*).

In a discussion concerning cofactors and hydroxylation reactions it would be remiss to confine the subject to aromatic compounds. Non-heme iron proteins participate in the hydroxylation of octane (*14*), camphor (*15*), and steroid (*16*) molecules. These low-molecular-weight proteins apparently take part in the transfer of electrons from $NADH_2$ to the enzymes catalyzing oxygen fixation into the substrate molecule. Since few enzymes which hydroxylate the aromatic nucleus have been purified, it seems possible that non-heme iron proteins may be involved in the hydroxylation of aromatic compounds.

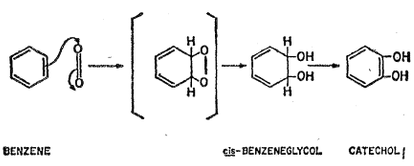


Fig. 2. Alternative pathway of catechol formation from benzene.

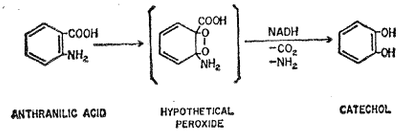


Fig. 3. Mechanism of catechol formation from anthranilic acid.

In view of the considerable interest in the degradation of aromatic compounds, it is surprising to find that relatively little is known about benzene metabolism. Marr and Stone (*17*) have detected catechol chromatographically in the culture medium of a *Pseudomonas* sp. that utilized benzene as sole source of carbon for growth. Phenol was excluded as an intermediate in benzene degradation since benzene-grown cells failed to metabolize this compound. Hypothetical pathways for the formation of catechol from benzene are shown in Fig. 1. Sequence 1 involves epoxidation of the aromatic ring followed by hydrolysis to form *trans*-benzeneglycol (1,2-dihydroxy-3,5-cyclohexadiene). Enzymatic dehydrogenation of *trans*-benzeneglycol results in catechol formation. This pathway is analogous to the first stages of the microbial degradation of naphthalene. Although an epoxide has not been identified in the oxidation of naphthalene, Walker and Wiltshire (*18*) isolated *trans*-dihydronaphthalenediol [(+)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene] from cultures of a *Bacillus* sp. This compound is assumed to arise from an epoxide precursor (*19*). Griffiths and Evans (*20*) prepared a cell extract that oxidized naphthalene to *trans*-naphthalenediol in the presence of $NADPH_2$ and ferrous ions.

Recently we isolated an organism, identified as *Pseudomonas putida*, which utilizes toluene as sole source of carbon for growth (*8*). Cells grown on toluene also metabolize benzene. Surprisingly these cells metabolize *cis*-benzeneglycol but not the *trans*-isomer. Cell extracts oxidized benzene when supplied with $NADH_2$, ferrous ions, and a reducing agent. Radioisotope-trapping experiments implicated *cis*-benzeneglycol as an intermediate in the formation of catechol from benzene, suggesting that an epoxide is not an intermediate in benzene degradation by *Pseudomonas putida*. An alternative reaction sequence is shown in Fig. 2.

Table 1. Catechol and protocatechuic acid as central intermediates in the degradation of aromatic compounds.

Compounds metabolized via	
Catechol	Protocatechuic acid
Benzoic acid	*m*-Cresol
Salicylic acid	*p*-Cresol
Phenol	*p*-Hydroxybenzoic acid
Naphthalene	*p*-Hydroxymandelic acid
Phenanthrene	*p*-Aminobenzoic acid
Anthracene	Phthalic acid
Mandelic acid	
o-Cresol	
Benzene	

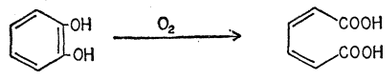
An epoxide has been postulated as an intermediate in the conversion of anthranilic acid to catechol. This reaction is catalyzed by an enzyme isolated from a *Pseudomonas* sp. (*21*). The enzyme is synthesized by the organism during growth on tryptophan. However, recent studies with isotopic oxygen have shown that both atoms of the oxygen molecule are incorporated into the aromatic substrate (*22*). As a result of these observations a cylic peroxide intermediate has been proposed as a product of anthranilic acid oxidation (Fig. 3).

Recent studies have revealed a mechanism for the hydroxylation of phenylalanine (*23*). When a purified preparation of bacterial phenylalanine hydroxylase was incubated with L-phenylalanine tritiated in the *para* position, the tyrosine produced still contained a considerable amount of tritium. Similar results were obtained when phenylalanine deuterated in the *para* position was used as a substrate (*24*). The reaction sequence in Fig. 4 has been proposed to explain the retention of isotopic material. This mechanism assumes that the hydroxylation is electrophilic. The strength of the carbon-tritium or carbon-deuterium bond is substantially greater than that of the carbon-hydrogen bond. Consequently, when R is deuterium or tritium, a large proportion of isotopic tyrosine is produced.

It is apparent that diverse mechanisms are utilized by microorganisms to prepare different aromatic compounds for ring fission. Hayaishi (*3*) has proposed the terms monooxygenases for enzymes that incorporate one atom of oxygen into a substrate molecule, and dioxygenases for enzymes that incorporate both atoms of the oxygen molecule. Dioxygenases function principally in the enzymatic fission of the aromatic nucleus. However, anthranilate hydroxylase and possibly benzene hydroxylase are dioxygenases.

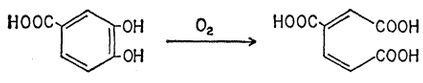
Ring Fission

Catechol and protocatechuic acid are metabolic intermediates in the microbial degradation of many different aromatic compounds. Consequently, these dihydroxylated benzene derivatives have been the objects of intensive investigation. Hayaishi and Hashimoto (*25*) isolated an enzyme, catechol-1,2-dioxygenase, that catalyzed the incorporation of molecular oxygen into the catechol molecule. The reaction product was identified as *cis,cis*-muconic acid. An analogous reaction was reported for the metabolism of protocatechuic acid (*26*).



Catechol — *cis,cis*-Muconic acid

The enzyme protocatechuic acid-3,4-dioxygenase cleaves this acid between the hydroxyl groups to form β-carboxy-*cis,cis*-muconic acid.



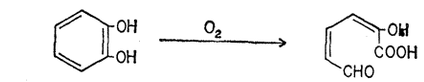
Protocatechuic acid — β-Carboxy-*cis,cis*-muconic acid

Both enzymes have been purified, and each contains ferric iron at its active center (*4*). It is interesting that the removal of iron requires a reducing agent and a chelating reagent specific for ferrous iron. The resulting iron-free protein is inactive, and activity cannot be restored by the addition of ferric iron. Reactivation of the enzyme requires the addition of divalent iron and an oxidizing agent. The iron in both enzymes exists in the trivalent state. Electron spin resonance studies revealed that, when both enzymes combine with their respective substrates, the iron at the active center is reduced.

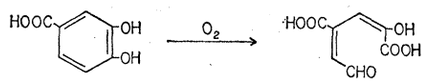
An alternative enzymatic cleavage of catechol was reported by Dagley and Stopher (*27*). A *Pseudomonas* sp. which utilized *o*-cresol as sole source of carbon for growth, oxidized catechol to α-hydroxymuconic semialdehyde. The enzyme catalyzing this reaction is catechol-2,3-dioxygenase. Growth of a different *Pseudomonas* sp. on *p*-cresol induced the formation of an enzyme, protocatechuic acid-4,5-dioxygenase, which oxidized protocatechuic acid to α-hydroxy-γ-carboxymuconic semialdehyde (*28*).

Table 2. Aromatic compounds and their ring-fission substrates.

Aromatic compound	Ring-fission substrate
Toluene or *o*-cresol	2,3-Dihydroxytoluene
β-Phenylpropionic acid	2,3-Dihydroxy-β-phenylpropionic acid
Phenylacetic acid	3,4- or 2,5-Dihydroxyphenylacetic acid
Naphthalene	1,2-Dihydroxynaphthalene
Anthracene	1,2-Dihydroxyanthracene
Phenanthrene	3,4-Dihydroxyphenanthrene
3-Hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione	3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione
Estrone	4-Hydroxyestrone



Catechol — α-Hydroxymuconic semialdehyde



Protocatechuic acid — α-Hydroxy-γ-carboxymuconic semialdehyde

Catechol-2,3-dioxygenase has been extracted and crystallized from cells of *Pseudomonas arvilla* (*29*). The enzyme is extremely sensitive to oxygen and is easily inactivated in the presence of air. However, low concentrations of acetone or ethanol protect the enzyme from atmospheric inactivation. This unexplainable observation facilitated the purification and crystallization of the enzyme.

Crystallized catechol-2,3-dioxygenase contains ferrous iron at its active center. Electron spin resonance studies revealed that, in the presence of catechol and oxygen, the iron is converted to the ferric state. These observations, considered in conjunction with other physicochemical studies, led Hayaishi to propose the reaction mechanism shown in Fig. 5. In this system, an oxygen molecule combines with the iron in the enzyme to form a perferryl ion. After catechol binds to the ferric oxygen to form a ternary complex, an electron from the phenolic oxygen reduces the iron with the concomitant release of an oxygen radical. The activated oxygen and catechol molecules react to form a peroxide intermediate which undergoes molecular rearrangement to form α-hydroxymuconic semialdehyde. The entire sequence of events is pictured as occurring in a complex of iron, oxygen, and catechol on the enzyme surface. At the present time, this is the most satisfactory reaction mechanism proposed for the action of a dioxygenase.

Reaction Sequences Initiated by Dioxygenases

Catechol and protocatechuic acid undergo enzymatic ring cleavage with the formation of *cis,cis*-muconic acid and β-carboxy-*cis,cis*-muconic acid, respectively. Ornston and Stanier (*30*) have reinvestigated the metabolism of these ring-fission products. Their studies with *Pseudomonas putida* demonstrated

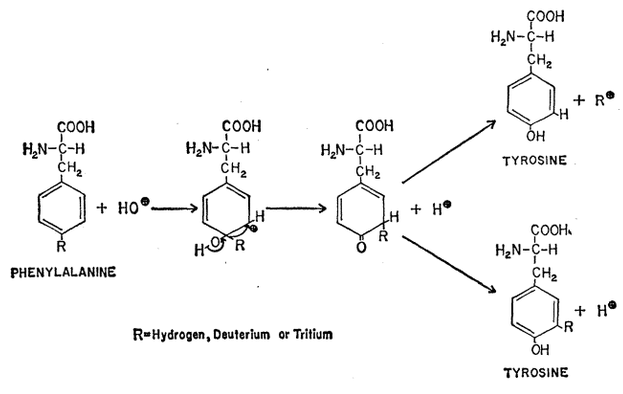


Fig. 4 (left). Mechanism for the hydroxylation of phenylalanine.

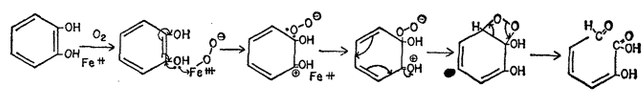


Fig. 5 (above). Proposed mechanism for the action of catechol-2,3-dioxygenase.

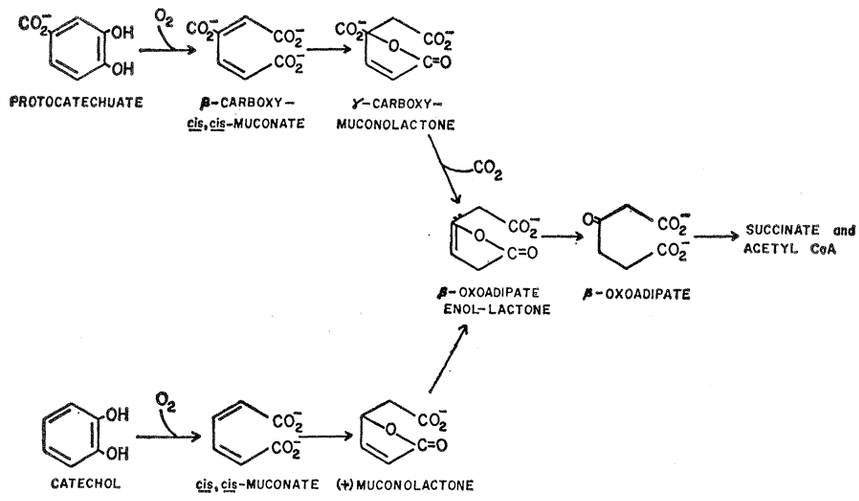


Fig. 6. Degradation of catechol and protocatechuic acid by *Pseudomonas putida.*

that β-oxoadipic acid enol-lactone (γ-carboxymethyl-Δ^{β}-butenolide) is a common intermediate in the sequence of reactions initiated by catechol-1,2-dioxygenase and protocatechuic acid-3,4-dioxygenase. In addition a new intermediate compound, γ-carboxymuconolactone (γ-carboxy-γ-carboxymethyl-Δ^{β}-butenolide) was identified in the protocatechuic acid pathway.

It is now established that the three reactions responsible for the conversion of protocatechuic acid to β-oxoadipic acid enol-lactone are analogous to the reactions utilized to convert catechol to β-oxoadipic acid enol-lactone (Fig. 6). The further metabolism of this common intermediate compound occurs by hydrolysis of the lactone ring to form β-oxoadipic acid, which is cleaved after activation to yield acetyl coenzyme A and succinic acid.

An elegant study of the regulation of enzyme synthesis in the catechol and protocatechuic acid pathways indicates that two groups of enzymes are controlled by two different compounds (*31*). The enzymes of the catechol pathway, *cis,cis*-muconate lactonizing enzyme and muconolactone isomerase, appear to be induced by *cis,cis*-muconic acid. Synthesis of β-carboxy-*cis,cis*-muconate lactonase and γ-carboxymuconolactone decarboxylase, enzymes of the protocatechuic acid pathway, is induced by β-oxoadipic acid or β-oxoadipylcoenzyme A. The last-named compound also induces the formation of β-oxoadipate enol-lactone hydrolase which is a common enzyme for both pathways. Thus, cells induced to metabolize catechol also contain enzymes for the metabolism of two compounds in the protocatechuic acid pathway. The reverse situation is not observed since *cis,cis*-muconic acid induces the two analogous enzymes in the catechol pathway.

Although *Moraxella lwoffii* uses the same enzymatic steps for the degradation of both catechol and protocatechuic acid, the regulation of enzyme synthesis is entirely different. All four enzymes that convert protocatechuic acid to β-oxoadipic acid appear to be under coordinate control. The inducing agent for these enzymes is probably protocatechuic acid.

The reaction sequences initiated by catechol-2,3-dioxygenase and protocatechuic acid-4,5-dioxygenase lead to the formation of highly reactive carbonyl compounds. It is a characteristic of all compounds, formed by fission adjacent to one of the two hydroxyl groups, that they can undergo nonenzymatic ring closure in the presence of ammonium ions. This is illustrated in Fig. 7, where the ring-fission products from catechol and protocatechuic acid form the pyridine derivatives picolinic and 2,4-lutidinic acids, respectively. Analogous pyridine carboxylic acids are formed from the ring-fission products from 3,4-dihydroxy-9,10-seco-androsta-1,3,5-(10)-triene-9,17-dione (*9*), 4-hydroxyestrone (*32*), 2,3-dihydroxy-β-phenylpropionic acid (*33*), and 3,4-dihydroxyphenylacetic acid (*34*).

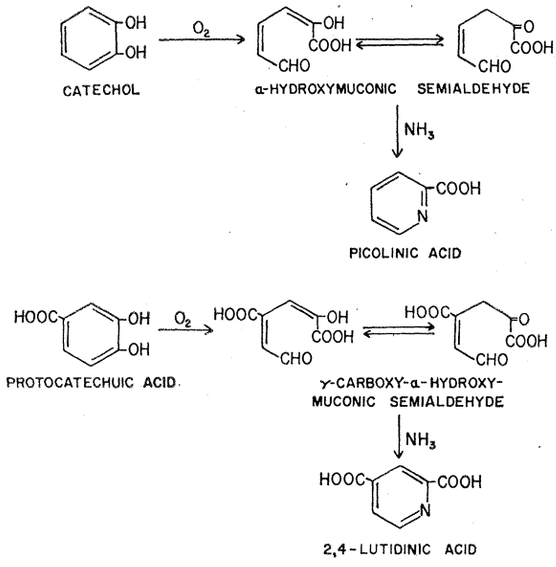


Fig. 7. (left). Ring-fission products from catechol and protocatechuic acid and their respective pyridine acids.

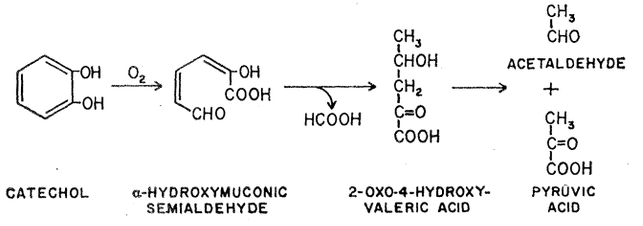


Fig. 8 (above). Conversion of catechol into acetaldehyde and pyruvic acid by a species of *Pseudomonas.*

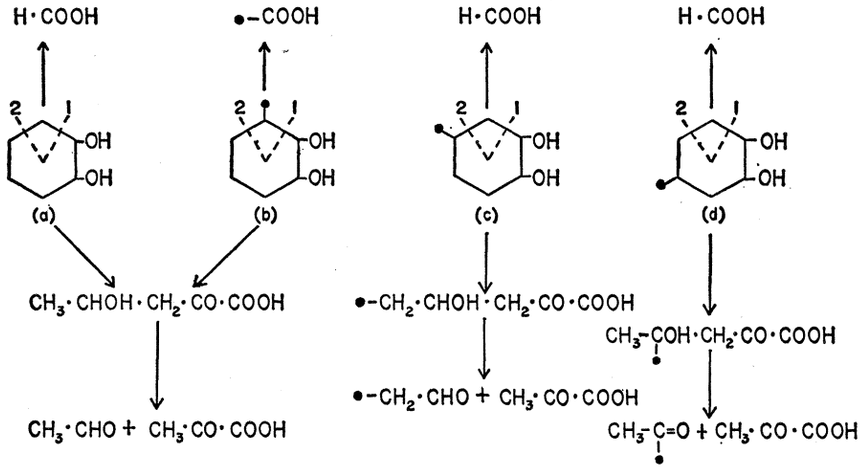


Fig. 9. General pathway for the degradation of dihydroxylated aromatic compounds. For explanation, see text.

The reaction sequence for the metabolism of catechol by a *Pseudomonas* sp. is shown in Fig. 8 (*35*). It can be seen that α-hydroxymuconic semialdehyde is formed with the consumption of 1 mole of oxygen per mole of catechol. Further metabolism of the ring-fission compound results in the formation of 2-oxo-4-hydroxyvaleric and formic acids. The former subsequently undergoes aldol cleavage to form pyruvic acid and acetaldehyde. Analogous reaction sequences were observed during the microbial degradation of several dihydroxy compounds.

As a result of these observations, Dagley and his colleagues have proposed a general metabolic scheme for the microbial degradation of aromatic compounds (*2*). In the proposed sequence (Fig. 9) the aromatic nucleus is opened by a 2,3-dioxygenase along broken line 1. The next cleavage is shown by line 2. Substituents on the aromatic ring are represented by the closed circles. Thus, Fig. 9a represents the degradation of catechol and Fig. 9b shows the production of a substituted formic acid. When 3-methylcatechol or 2,3-dihydroxy-β-phenylpropionic acid are the substrates, the acids liberated are acetic acid and succinic acid, respectively. Substitution of the aromatic ring at points other than position 3 results in the incorporation of the substituent group into a γ-hydroxy oxoacid. Aldol cleavage of the substituted hydroxyacid produces a substituted carbonyl compound and pyruvic acid. Examples of this type of metabolism are shown in Fig. 9, c and d, and may be observed in the degradation of 4-methylcatechol, resulting in the formation of propionaldehyde and pyruvic acid and also in the formation of two molecules of pyruvic acid from protocatechuic acid. The general scheme has been successfully applied to the microbial degradation of naphthalene (*36*) and aromatic steroids (*9*). Work in our laboratory suggests that the metabolic pathways utilized by bacteria to degrade several aromatic hydrocarbons will also follow the predicted pathway.

Since microorganisms do not live by a rigid set of rules, it is not surprising to find exceptions to the proposed scheme. Thus, the metabolism of kynurenic acid which undergoes an analogous 2,3-fission is not accommodated by the general pathway (*37*). Also, a different pathway for the degradation of catechol has been reported (*38*). In this sequence of events, the aldehyde group of α-hydroxymuconic semialdehyde is oxidized to a carboxyl group. Subsequent decarboxylation then produces 2-oxo-4-hydroxyvaleric acid. No doubt more exceptions will be found in the future. Nevertheless, the general scheme provides a practical working model for the prediction of possible intermediates in the metabolism of various aromatic compounds. As such it should be a valuable tool to the microbial physiologist.

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