The Microbiological Degradation of Aromatic Compounds

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The breakdown of aromatic compounds by ring cleavage is an essential biochemical step in Nature's 'carbon' cycle and is performed by several kinds of micro-organism. Bacteria are the most versatile in this respect, but several yeasts and fungi are able to degrade a more limited range of benzenoid structures (see list of Reviews on p. 183).

Among the eubacteria, representatives of the families Coccaceae, Mycobacteriaceae, Pseudomonadaceae, Spirillaceae, Bacteriaceae and Bacillaceae are active in this respect. The yeasts *Oospora*, *Candida*, *Debaromyces*, *Pichia* and *Saccharomyces* grow in media with catechol as sole carbon source. Certain higher fungi, e.g. *Aspergillus*, *Penicillium* and *Neurospora* attack aromatics and a variety of soil and wood-rotting fungi dissimilate the aromatic polymer lignin, as well as other plant phenolics. In all the above cases of aromatic ring metabolism, molecular oxygen is an obligatory oxidant.

The photosynthetic bacteria *Rhodospseudomonas* and some *Rhodospirillum* strains utilize benzoate under strictly anaerobic conditions in the light. Finally, there exists an anaerobic type of aromatic ring metabolism—e.g. the so-called methane fermentation of benzoate, although it is doubtful whether these methane bacteria are in pure culture.

These microbes produce, mostly as a result of induction, a whole sequence of enzymes which convert aromatic substrates into an *ortho* or *para* dihydroxy-phenol derivative, followed by cleavage of the ring to aliphatic acids; these ring fission products are then funnelled into the Krebs cycle through a variety of pathways, depending on the organism and cultural conditions.

The distinctive biochemical step is ring cleavage. There are many instances of microbes being able to modify aromatic compounds, either by hydroxylation, or elimination of substituent groups, without necessarily causing fission. In the case of the aromatic amino acids, phenylalanine, tyrosine and tryptophan, pathways of metabolism exist that have much in common in all forms of life. It is only possible to sketch the salient features of microbial aromatic ring metabolism here, since the topic has, of recent years, greatly increased in scope.

(1) *Aromatic hydroxylating enzymes*

Aromatic hydrocarbons undergo a perhydroxylation to the *trans*-dihydro-arene diols, which are then dehydrogenated to the *o*-dihydroxyphenol prior to ring fission, thus:

A similar system exists in liver microsomes. The bacterial enzymes are extremely labile and difficult to separate; parts of this scheme are therefore still hypothetical. The corresponding 'diol' intermediate has been isolated from cultures of naphthalene (Walker & Wiltshire, 1953) and phenanthrene (Colla, Fiecchi & Treccani, 1959); evidence for its formation also exists in the case of benzene (Marr & Stone, 1959, 1961) and anthracene (Colla *et al.* 1959). Ayengar, Hayaishi, Nakajima & Tomida (1959) studied reaction III, using sonic extracts of *Aerobacter aerogenes* known to contain a specific myoinositol dehydrogenase, but conveniently lacking the enzymes catalysing reactions I, II, and IV. With *trans*-3,5-cyclohexadiene-1,2-diol as substrate, catechol was formed; NAD and a regenerating system were necessary for maximal activity.

The perhydroxylation step is Fe^{2+} dependent, and to account for the invariable formation of *trans*-dihydrodiols, the enzyme + oxygen + substrate complex is visualized as being of the epoxide type; second-order nucleophilic attack by a hydroxyl anion would yield the *trans*-isomer.

Polynuclear aromatic hydrocarbons attach to the relevant bacterial enzymes at C-C bonds of high electron density (the K regions of Pullman & Pullman, 1955). If they have a linear configuration, e.g. naphthalene, anthracene, attachment and ring splitting takes place on the same ring. Angular aromatic compounds such as phenanthrene afford attachments to an enzyme at a bond in a ring other than the one containing the ring-splitting site (Rogoff, 1962).

Benzoate, salicylate and anthranilate are common intermediates in degradative pathways of more complex aromatic compounds. These acids are converted to catechol by bacterial oxidases before ring fission; recently much light has been shed on these particular enzymes. Ichihara, Adachi, Hosokawa & Takeda (1962) prepared active cell-free systems which oxidatively decarboxylated these acids and caused ring cleavage of the resulting catechol. If *meta*-substituted benzoates were used as substrates, the oxidase preparations converted them to the relevant catechols only, since the specificity of the fission enzymes prevented them from being metabolized further; in this way they were able to study the initial enzyme reactions. Another successful approach has been the purification of the relevant enzyme systems. Thus Hosokawa, Nakagawa & Takeda (1961) fractionated crude anthranilate oxidase preparations into two indispensable protein components. Katagiri, Yamamoto & Hayaishi (1962) separated salicylate hydroxylase activity from the catechol cleavage enzyme. Results show these enzymes to be Fe^{2+} -dependent requiring NADH or NADPH (and regenerating systems, e.g. ethanol and alcohol dehydrogenase). In the case of salicylate hydroxylase, an absolute requirement for flavin adenine dinucleotide (FAD) was demonstrated in addition.

The mode of action of reduced pyridinenucleotides is not yet clear, but as suggested by Mason (1957) for a mixed function oxidase, one atom of oxygen might be

used to hydroxylate the substrate, and the second be reduced by the NADH (or NADPH). A variety of electron donors function in different hydroxylating systems, e.g. NADH, NADPH, FADH₂ in the case of the bacterial enzymes; ascorbic acid with phenolases; tetrahydropteridine in the phenylalanine hydroxylase of liver, although NADPH serves as a reductant indirectly to reduce the oxidized pteridine derivative.

Brief mention must be made at this point of the fate of the other monohydroxybenzoates. Yano & Arima (1958) succeeded in extracting from some bacteria hydroxylase A, which converted *m*-hydroxybenzoate to protocatechuate, and from other organisms a hydroxylase B, which gave gentisate. Both hydroxylases required NADPH as co-factor. The subsequent pathways of these two dihydroxybenzoates differ (Figs. 1, 2). *p*-Hydroxybenzoate gives protocatechuate in all organisms studied.

Fig. 1. Oxidative fission of *o*-dihydroxyphenols by micro-organisms.

(2) *Aromatic ring-cleavage enzymes**

The enzyme molecule contains Fe²⁺ and sulphydryl groups, the integrity of both being essential for activity; molecular oxygen is obligatory.

In the case of *o*-dihydroxyphenol derivatives, two distinct cleavage enzymes are known. Their action may be illustrated with respect to catechol and protocatechuate, two central aromatic intermediates (Fig. 1).

(a) Pyrocatechase (catechol 1,2-oxygenase) and protocatechuic acid oxidase (protocatechuate 3,4-oxygenase) open the C-C bond between the two hydroxyls.

* Dagley, Evans & Ribbons (1960).

The ring fission product, a *cis-cis* muconic acid, is then lactonized by a Mn^{2+} dependent enzyme; hydrolytic cleavage of this to β -oxoadipate is achieved by a delactonizing enzyme. An enzymic C4–C2 split of this oxo-acid in the presence of coenzyme A and catalytic amounts of succinyl-coenzyme A provides the substrates for the tricarboxylic acid cycle, thus:

(b) Catechol 2,3-oxygenase (metapyrocatechase) and protocatechuate 4,5-oxygenase open the C–C bond adjacent to a hydroxyl. The muconic semialdehyde so formed can undergo a non-enzymic reaction with NH_4^+ ions to give a pyridine nucleus, or be metabolized to Krebs cycle acids, one of which is certainly pyruvate. In their announcement of the new catechol pathway, Dagley & Stopher (1959) suggested a route of metabolism of α -hydroxymuconic semialdehyde to pyruvate without gaseous exchange; subsequently, Nishizuka, Ichiyama, Nakamura & Hayaishi (1962) have suggested the following route:

It is interesting to note that Gholson *et al.* (1962) have recently implicated α -hydroxymuconic semialdehyde in the catabolism of tryptophan by mammalian liver. Ribbons & Evans (1962) adduced evidence in favour of pyruvate and malate as the products of protocatechuate metabolism via γ -carboxy- α -hydroxymuconic semialdehyde.

In the case of *p*-dihydroxyphenols, rupture of the bond between the carbon atom bearing a hydroxyl and an adjacent carbon atom carrying a side chain or carboxyl, occurs. For homogentisate, this method is common to micro-organisms (Chapman & Dagley, 1962) and mammals, being the pathway of tyrosine metabolism. Both homogentisicase and gentisicase give rise initially to the aliphatic oxo-acids with the *cis* (maleyl) configuration, but a glutathione dependent isomerase converts them to the *trans* (fumaryl) isomers; a hydrolase then catalyses fission of these to fumarate and acetoacetate, and fumarate and pyruvate respectively (Fig. 2).

There are two routes of tryptophan metabolism found among Pseudomonads. The 'anthranilate' pathway involves oxidative fission of the pyrrol ring to formyl-kynurenine, elimination of the formyl group and a non-oxidative cleavage of the kynurenine to alanine and anthranilate (subsequently to be metabolized via catechol). In the 'quinoline' pathway, kynurenic acid is formed, which is then hydroxylated in the C7, 8, positions; fission of the aromatic nucleus apparently occurs in two ways. Hayaishi & collaborators (Kuno *et al.* 1961) initially suggested a split between the two hydroxyls, then revised this view (Kojima, Itada & Hayaishi, 1961) in favour of cleavage between C8 and the angular C atom. The substituted pyridine rings so formed give rise to glutamate in both cases.

Mechanism of action of bacterial oxygenases. These all belong to the class of enzymes called 'oxygen transferases' by Mason (1957). A tracer study with ^{18}O proves that the two oxygen atoms in the ring fission product are derived from molecular oxygen (Hayaishi, Katagiri & Rothberg, 1957). It is thought that the first step is the formation of an oxyferro-oxygenase. Electrons have been donated to the oxygen from the iron, allowing the O—O bond to be weakened (Ingraham, 1962);

association of the perferryl complex with the *o*-dihydroxyphenol group leads to a redistribution of electrons and fission of the C—C bond. Oxygenases are labile in oxygen; Taniuchi *et al.* (1962) were able to restore catechol 2,3-oxygenase (meta-pyrocatechase) activity with sodium borohydride (and other reducing agents to a certain extent) best under anaerobic conditions. FADH₂, FMN and riboflavin in the presence of NADH were also effective, and they suggest a physiological role for flavin coenzymes in reductive enzyme reactivations.

Fig. 2. Oxidative fission of *p*-dihydroxyphenols by micro-organisms and mammals.

(3) Fate of substituent groups on monocyclic aromatic rings before cleavage

There are obviously a great many biochemical reactions which ring-splitting micro-organisms perform on substituted benzenoid structures before nuclear fission. A few of these may be illustrated.

(i) Aromatic methyl ethers are demethylated to the corresponding hydroxy-derivative. Semicarbazide traps the methoxyl C as formaldehyde; in its absence it is oxidized terminally to CO₂ (Woodings, 1961).

(ii) Methyl substituents may be oxidized step-wise to the carboxylic acid (e.g. the cresols), but in some cases it remains intact until after ring fission (e.g. MCPA metabolism, Evans, Gaunt & Davies, 1961).

(iii) Chloro-substituents usually remain on the ring unless removal is obligatory for the introduction of hydroxyls, which are necessary for fission.

(iv) Nitro and sulphonic acid groups can be eliminated and replaced by hydroxyl.

(v) A carboxyl may be oxidatively decarboxylated; in some cases anaerobic decarboxylation occurs, e.g. of 4,5-dihydroxyphthalate to protocatechuate (Ribbons & Evans, 1960).

(vi) Aliphatic side-chains are dealt with in a variety of different ways. They may be eliminated, by β -oxidation (Webley, Duff & Farmer, 1955) or other mechanisms familiar to biochemistry, or even remain intact—e.g. phenylpropionic acid (Coulson & Evans, 1959; Dagley & Chapman, 1961, private communication).

(4) *Metabolism of polynuclear aromatic hydrocarbons*

Perhydroxylation followed by dehydrogenation produces 1,2-dihydroxynaphthalene, 1,2-dihydroxyanthracene, and 3,4-dihydroxyphenanthrene from their parent hydrocarbons. The actual site of cleavage in the naphthalene nucleus is the bond between the angular C and C1 which also carries a hydroxyl (Davies & Evans, 1962). There is strong evidence that it takes place at the analogous bond in 1,2-dihydroxyanthracene and 3,4-dihydroxyphenanthrene (Fernley & Evans, 1958; Griffiths & Evans, 1963, personal communication).

The case of naphthalene will serve to illustrate this pathway: A Fe^{2+} oxygenase converts 1,2-dihydroxynaphthalene to *o*-hydroxybenzalpyruvate; a hydrolytic split converts this to salicylaldehyde and pyruvate; a NAD dependent dehydrogenase produces salicylate from the aldehyde; a salicylate hydroxylase gives catechol, which is ruptured to α -hydroxymuconic semialdehyde by the catechol 2,3-oxygenase also present in the cell-free extract.

(5) *Anaerobic metabolism of certain aromatic compounds*

Hitherto, we have considered only the aerobic metabolism of aromatic compounds by micro-organisms. Although much less is known about them, certain derivatives of benzene are dissimilated under anaerobic conditions by some bacteria.

Photosynthetic bacteria require accessory hydrogen donors for anaerobic photosynthesis in light; although evolution of oxygen has never been observed, an oxidizing entity of sufficient positive potential to perform the peroxidation of benzoate is produced by several strains of non-sulphur photosynthetic bacteria, e.g. *Rhodospseudomonas* and *Rhodospirillum*. According to Proctor & Scher (1960) sequential induction evidence supports the following tentative scheme:

Clarke & Fina (1952) confirmed the utilization of benzoate by methane-producing cultures; catechol and protocatechuate could not be metabolized by these cultures, and exogenous CO_2 was not reduced mainly to methane. In subsequent tracer studies (Fina & Fiskin, 1960; Roberts, 1962) it was determined that the carboxyl-C and C4 of benzoate behaved the same as exogenous CO_2 . They were not reduced to methane primarily; on the other hand, C1 of the ring was converted almost entirely

to methane. Benzoate utilizing cultures did not produce methane and CO₂ from fumarate, acrylate or isobutyrate without very long adaptive lag periods. Cyclohexane carboxylic acid, butyric and propionic acids, however, produced gas readily with no lag period. Propionate, containing C4 of the ring, was isolated from the benzoate culture liquor; it did not contain C1 or C7. One can thus speculate that there is a preliminary saturation of the ring followed by a rupture between C1 and C2 before propionate is released. No other organic acid has yet been isolated.

REVIEWS

- BEERSTECHEER, E. (1954). *Petroleum Microbiology*. New York: Elsevier Press Inc.
- EVANS, W. C. (1958). Metabolism of aromatic compounds by lower plants, phenols, quinones, and the problem of the biogenesis of benzene nuclei. *Handb. Pfl. Physiol.* **10**, 454.
- HAPPOLD, F. C. (1950). The oxidation of aromatic rings by micro-organisms in metabolism. *Symp. biochem. Soc.* **5**, 85.
- HAYAISHI, O. (1957). Studies on oxygenases. Topic II. Enzyme systems of hydrogen, oxygen and electron transfer. *Proc. int. Symp. Enzyme Chem. I.U.B. Symp. Series*, **2**, 207. Tokyo and Kyoto.
- HAYAISHI, O. (1962). *Oxygenases*. New York Academic Press.
- IND. & ENG. CHEM. SYMP. (1956). *Purifying Chemically Polluted Waters*, **48**, 1403.
- ROGOFF, M. H. (1961). Oxidation of aromatic compounds by bacteria. *Advanc. appl. Microbiol.* **3**, 193.
- STANIER, R. Y. (1955). Cleavage of aromatic rings with eventual formation of β -ketoadipic acid. *Meth. Enzymol.* **2**, 273.
- TAUSSON, W. O. (1950). *Basic Principles of Plant Bioenergetics*. (Collected works.) Ed. N. A. Maximov. U.S.S.R.: Acad. Sci.
- TRECCANI, V. (1963). Microbial degradation of hydrocarbons. *Progress in Industr. Microbiol.* **4** (in the Press).
- WOODCOCK, D. (1960). *The Metabolism of Aromatic Compounds by Fungi. Phenolics in Plants in Health and Disease*, p. 75. London: Pergamon Press.
- ZOBELL, C. E. (1946). Action of micro-organisms on hydrocarbons. *Bact. Rev.* **10**, 1.
- ZOBELL, C. E. (1950). Assimilation of hydrocarbons by micro-organisms. *Advanc. Enzymol.* **10**, 443.

REFERENCES

- AYENGAR, P. K., HAYAISHI, O., NAKAJIMA, M. & TOMIDA, I. (1959). Enzymic aromatization of 3,5-cyclohexadiene-1,2-diol. *Biochim. biophys. Acta*, **33**, 111.
- BEHRMAN, E. J. & TANAKA, T. (1959). The quinoline pathway of tryptophan oxidation by *Pseudomonas*: the initial steps in the oxidation of kynurenine acid. *Biochem. biophys. Res. Comm.* **1**, 257.
- CHAPMAN, P. J. & DAGLEY, S. (1962). Oxidation of homogentisic acid by cell-free extracts of a *Vibrio*. *J. gen. Microbiol.* **28**, 251.
- CLARKE, F. M. & FINA, L. R. (1952). The anaerobic decomposition of benzoic acid during methane fermentation. *Arch. Biochem.* **36**, 26.
- COLLA, C., FIECCHI, A. & TRECCANI, V. (1959). Ricerche sul metabolismo ossidativo microbico dell'antracene e del fenantrene. Nota II. Isolamento e caratterizzazione del 3,4-diidro-3,4-diossifenantrene. *Ann. Microbiol. e Enzimol.* **9**, 87.
- COULSON, C. B. & EVANS, W. C. (1959). Microbiological degradation of *trans*-cinnamic acid by soil *Pseudomonads*. *J. Soc. chem. Ind., Lond.* p. 543.
- DAGLEY, S., EVANS, W. C. & RIBBONS, D. W. (1960). New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. *Nature, Lond.* **188**, 560.
- DAGLEY, S. & STOPHER, D. A. (1959). A new mode of fission of the benzene nucleus by bacteria. *Biochem. J.* **73**, 16P.

- DAVIES, J. I. & EVANS, W. C. (1962). Ring fission of the naphthalene nucleus by certain soil Pseudomonads. *Biochem. J.* **85**, 21 P.
- EVANS, W. C., GAUNT, J. K. & DAVIES, J. I. (1961). The metabolism of the chlorophenoxycetic acid herbicides by soil micro-organisms. *Proc. 5th int. Congr. Biochem., Moscow*, Sec. **14**, 306.
- FERNLEY, H. N. & EVANS, W. C. (1958). Oxidative metabolism of polycyclic hydrocarbons by soil Pseudomonads. *Nature, Lond.* **182**, 373.
- FINA, L. E. & FISKIN, A. M. (1960). The anaerobic decomposition of benzoic acid during methane fermentation. II. Fate of carbons 1 and 7. *Arch. Biochem.* **91**, 163.
- GHOLSON, R. K., NISHIZUKA, Y., ICHIYAMA, A., KAWAI, H., NAKAMURA, S. & HAYAISHI, O. (1962). New intermediate in the catabolism of tryptophan in mammalian liver. *J. biol. Chem.* **237**, PC 2043.
- HAYAISHI, O., KATAGIRI, M. & ROTHBERG, S. (1957). Studies on oxygenases; pyrocatechase. *J. biol. Chem.* **229**, 905.
- HOSOKAWA, K., NAKAGAWA, H. & TAKEDA, Y. (1961). Cofactor requirements for anthranilate oxidase. *J. Biochem., Tokyo*, **49**, 355.
- ICHIHARA, A., ADACHI, K., HOSOKAWA, K. & TAKEDA, Y. (1962). The enzymatic hydroxylation of aromatic carboxylic acids; substrate specificities of anthranilate and benzoate oxidases. *J. biol. Chem.* **237**, 2296.
- INGRAHAM, L. L. (1962). *Biochemical Mechanisms*. New York: Wiley and Sons Inc.
- KATAGIRI, M., YAMAMOTO, S. & HAYAISHI, O. (1962). Flavin adenine dinucleotide requirement for the enzymatic hydroxylation and decarboxylation of salicylic acid. *J. biol. Chem.* **237**, 2413.
- KOJIMA, Y., ITADA, N. & HAYAISHI, O. (1961). Metapyrocatechase: a new catechol-cleaving enzyme. *J. biol. Chem.* **236**, 2223.
- KUNO, S., TASHIRO, M., TANIUCHI, H., HORIBATA, K. & HAYAISHI, O. (1961). Enzymatic degradation of kynurenic acid. *Fed. Proc.* **20**, 3.
- MARR, E. K. & STONE, R. W. (1959). The bacterial oxidation of benzene. *Dissertation Abstr.* **20**, 460.
- MASON, H. S. (1957). Mechanisms of oxygen metabolism. *Advanc. Enzymol.* **14**, 79.
- NISHIZUKA, Y., ICHIYAMA, A., NAKAMURA, S. & HAYAISHI, O. (1962). A new metabolic pathway of catechol. *J. biol. Chem.* **237**, 268.
- PROCTOR, M. H. & SCHER, S. (1960). Decomposition of benzoate by a photosynthetic bacterium. *Biochem. J.* **76**, 33 P.
- PULLMAN, A. & PULLMAN, B. (1955). *Cancerisation par les substances chimiques et structure moleculaire*. Paris: Masson et Cie.
- RIBBONS, D. W. & EVANS, W. C. (1960). Oxidative metabolism of phthalic acid by soil Pseudomonads. *Biochem. J.* **76**, 310.
- RIBBONS, D. W. & EVANS, W. C. (1962). Oxidative metabolism of protocatechuic acid by certain soil Pseudomonads: A new ring-fission mechanism. *Biochem. J.* **83**, 482.
- ROBERTS, F. F. (1962). *Metabolism of benzoate by methane bacteria*. M.Sc. Thesis, Kansas State University, U.S.A.
- ROGOFF, M. (1962). Chemistry of oxidation of polycyclic aromatic hydrocarbons by soil Pseudomonads. *J. Bact.* **83**, 998.
- TANIUCHI, H., KOJIMA, Y., KANETSUNA, F., OCHIAI, H. & HAYAISHI, O. (1962). Mechanism of the autoxidation of oxygenases. *Biochem. biophys. Res. Comm.* **8**, 96.
- WALKER, N. & WILTSHIRE, G. H. (1953). The breakdown of naphthalene by a soil bacterium. *J. gen. Microbiol.* **8**, 273.
- WEBLEY, D. M., DUFF, R. B. & FARMER, V. C. (1955). Beta-oxidation of fatty acids by *Nocardia opaca*. *J. gen. Microbiol.* **13**, 361.
- WOODINGS, E. T. (1961). The microbial dissimilation of aromatic methyl ethers. *Dissertation Abstr.* **21**, 2438.
- YANO, K. & ARIMA, K. (1958). Metabolism of aromatic compounds by bacteria. II. *m*-hydroxybenzoic acid hydroxylase A and B; 5-dehydroshikimic acid, a precursor of protocatechuic acid; pathway from salicylic acid to gentisic acid. *J. appl. Microbiol.* **4**, 241.