Double hydroxylation reactions in microorganisms

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Abstract

The initial reactions involved in the degradation of aromatic hydrocarbons such as benzene, naphthalene, toluene, xylene, biphenyl, benzopyrene and benzoanthracene are discussed. The mechanism of oxidation of indole and pyrazon are described. Double hydroxylation reactions of benzoic acid, toluic acid, cumic acid and phthalic acid as well as the metabolic fate of aniline and anthranilic acid are illustrated. The enzymes involved in the double hydroxylation of the compounds in bacteria have been compared with those of the fungal system. The classification of oxygenases has been altered to define appropriately the double hydroxylating enzymes of the bacterial and fungal systems.

Key words : Double hydroxylations, aromatic hydrocarbons, benzoic acids, anilines, microbial metabolism.

1. Introduction

Oxygenases, which fix molecular oxygen into substrates, are widely distributed in nature and are generally involved in the metabolism of a variety of compounds. They participate in the biosynthesis and degradation of many metabolites, ranging from amino acids and lipids to porphyrins, vitamins and hormones. They also take part in the metabolic disposal of a variety of drugs and foreign substances.

In general, oxygen-rich compounds such as carbohydrates are not favourable substrates for oxygenases because these compounds usually have many reactive groups containing oxygen, such as hydroxyl, carbonyl or formyl and they do not need to be oxygenated further. On the other hand, lipids and aromatic compounds are often metabolized by oxygenases, presumably because these compounds are generally oxygen-deficient and need to be oxygenated in order to become biologically active or more soluble in water. Yet, because of the hydrophobic nature of lipids and aromatic compounds, molecular oxygen is the preferred hydroxylating agent rather than water. In contrast purines and pyrimidines with their hydrophobic ring systems, are usually hydroxylated by the addition of water, followed by dehydrogenation.

The part played by oxygenase in phenylalanine metabolism may serve as a useful example to demonstrate their physiological significance (Fig. 1). The map has been

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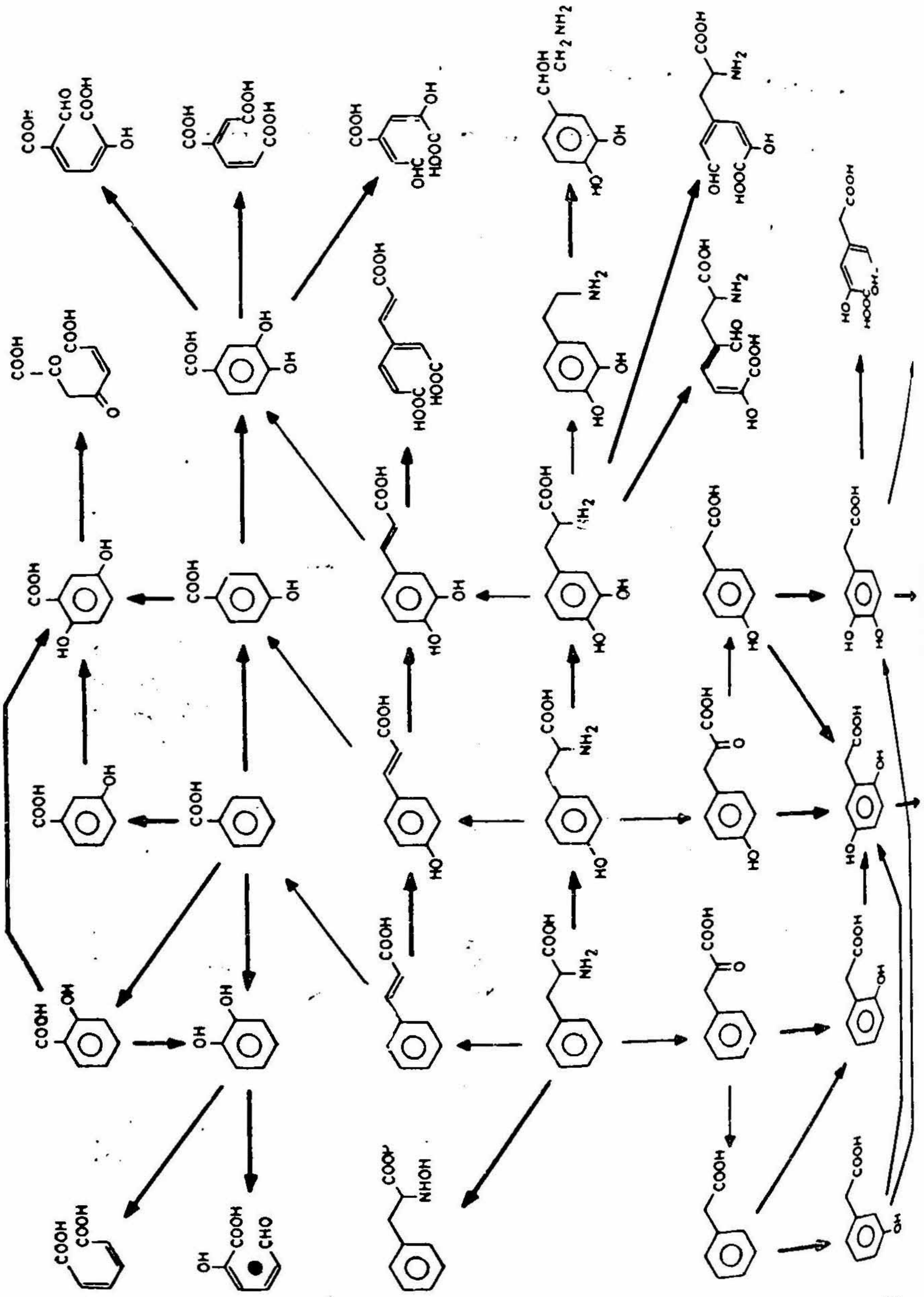


FIG. 1. Metabolism of phenylalanine.

This figure is an illustrative example of the role of oxygenases in the metabolism of phenylalanine. Bold arrows represent enzymes catalyzing oxygenation reactions.

worked out by several groups of investigators using various biological materials, namely, plants, animals as well as microorganisms. The map includes not only many degradative processes, but also biosynthetic pathways of physiologically important compounds. It is apparent that most of the oxidative steps are catalyzed by oxygenases rather than by oxidases or dehydrogenases.

Oxygenases are classified as shown in Table I

Table I

Classification of oxygenases

Oxygenase

A. *Monooxygenases*

1. Internal monooxygenase

2. External monooxygenase

   (*a*) Pyridine nucleotide-linked flavoprotein

   (*b*) Flavin-linked hemoproteins

   (*c*) Iron sulfur protein-linked hemoprotein

   (*d*) Pteridine-linked monooxygenase

   (*e*) Ascorbate-linked copper proteins

   (*f*) With another substrate as reductant

B. *Dioxygenase*

1. Intramolecular dioxygenase

   (*a*) Hemoproteins

   (*b*) Nonheme iron protein

   (*c*) Copper protein

   (*d*) Flavoprotein

2. Intermolecular dioxygenase

3. Miscellaneous

Cleavage of the benzene nucleus has always been thought of as the most important function of dioxygenases. However, recent studies on the metabolism of a number of compounds in microorganisms have shown the existence of another class of dioxygenases which carry out the double hydroxylation of the benzene ring, thus preparing an aromatic nucleus for attack by ring fission dioxygenases. The compounds which undergo dihydro-

xylation include hydrocarbons like benzene, naphthalene, anthracene, pyrene, phenanthrene, benzopyrene, toluene, xylene, biphenyl, aromatic acids like benzoic acid, phthalic acid, toluic acid, cumic acid, and aromatic amines like aniline, anthranilic acid and the recently studied herbicide, pyrazon.

Some of the double hydroxylating enzymes from bacteria, like anthranilate hydroxylase and benzoate oxygenase were discovered much earlier[1,2]. These enzymes have been classified under 'Miscellaneous' as shown in Table I. However, with the recent discovery of new enzyme systems catalyzing similar dihydroxylation reactions this class of dioxygenases requires to be separately classified. The classification of dioxygenases shown in Table I can be suitably altered to include the dihydroxylating dioxygenases as follows :

Dioxygenases

- Intermolecular
- Intramolecular
  - Ring cleaving
    - *a.* Hemoproteins
    - *b.* Nonheme iron proteins
    - *c.* Copper proteins
    - *d.* Flavoproteins
  - Ring hydroxylating
    - Ex : Anthranilate hydroxylase (bacteria)

Excellent review articles on various aspects of oxygenases that present a detailed account of their catalytic and biological functions are available[2-10]. In the following pages, discussion will be focussed on the recent developments in the area of ring hydroxylating dioxygenases from microorganisms.

2. Dihydroxylation of aromatic hydrocarbons

Aromatic hydrocarbons are found in nature as constituents of fossil fuels. The aromatic fraction generally constitutes less than 20% of total composition of most crude oil. Though it has been known for many years, that certain aromatic hydrocarbons are substrates for bacterial oxidation, the number of different structures known to be subject to microbial degradation represent a very small percentage of the aromatic hydrocarbons. Ranging from benzene to the polynuclear benzopyrene, the utilization of aromatic hydrocarbons by microorganisms has received much attention during the last decade. It has emerged that the degradation of aromatic hydrocarbons is initiated by a mechanism that differs from that of any monooxygenase reaction known to date, in so far as both the

atoms of molecular oxygen are incorporated simultaneously into the substrate as two hydroxyl groups[11, 12]. It appears that one molecule of oxygen is added to the aromatic ring to form a dioxetane intermediate and that the NADH required in the dihydroxylation is utilized for reducing the dioxetane to a dihydrodiol. Very few enzyme systems that catalyze the initial oxidation reaction mentioned above, have been isolated and studied. However, a number of intermediates isolated in recent years clearly illustrate the involvement of such enzyme systems in the oxidation of a number of aromatic compounds. In the following pages, the dihydroxylation reactions of aromatic hydrocarbons will be discussed.

Dihydroxylation of benzene

The bacterial oxidation of benzene appears to be confined to those species which are capable of forming the enzymes of the so-called aromatic pathway. The first step in the oxidation of benzene and other compounds is apparently unique and can be carried out only by a limited number of species. In the case of benzene, the enzymes for its degradation are inducible. Two organisms, a strain of *Pseudomonas* and *Mycobacterium* species capable of utilizing benzene as sole carbon source were isolated from soil by enrichment culture technique[13].

For many years, it was assumed that benzene is oxidized by bacteria through phenol. Park and William[12] showed the conversion of benzene to catechol without the intermediate formation of phenol. Haccius and his group[14, 15] isolated an organism capable of oxidizing benzene to catechol and identified it as *Nocardia corallina*. Nakajuna and Toida[16] and Mair and Stone[13] proposed a hypothesis that dihydrodiol is an intermediate in benzene degradation.

Gibson and his associates[17, 18] proposed a pathway for the degradation of benzene which involved a hypothetical cyclic peroxide intermediate. In mammalian system, epoxide is the proposed intermediate and *trans*-1,2-dihydro-1,2-dihydroxybenzene, the identified metabolite[19]. The scheme for the oxidation of benzene by bacterial and mammalian system is shown in Fig. 2.

Further Gibson *et al.*[20,21] showed that appropriately induced cultures of *Pseudomonas putida* oxidize benzene and ethylbenzene at equal rates. A mutant strain of this organism when grown on benzene accumulated *cis*-1,2-dihydroxycyclohexa-3,5-diene in the medium. The product was found to be identical with a synthetic sample of *cis*-1,2-dihydroxycyclohexa-3,5-diene. Experiments with isotopic oxygen showed the incorporation of both the atoms of molecular oxygen into *cis*-1,2-dihydroxycyclohexa-3,5-diene. The same compound was also isolated from a benzene utilizing *Moraxella*, B, by Hogn and Jaenicke[22]. Both the atoms of oxygen were shown to be incorporated into the above compound, simultaneously. The above results suggest that the initial reaction in benzene oxidation by *Pseudomonas* and *Moraxella*, involves a dioxygenation.

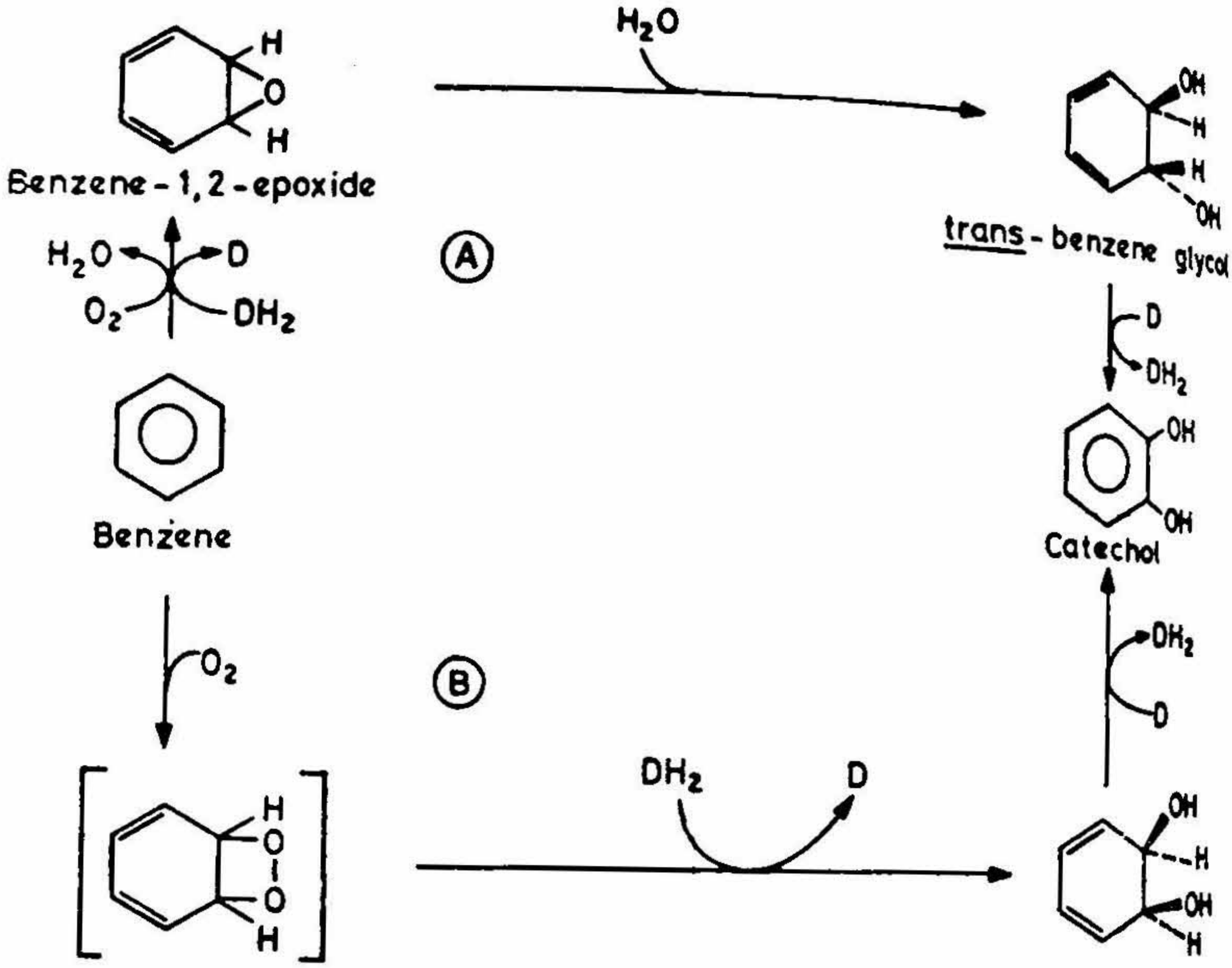


FIG. 2 (*a*). Metabolism of benzene (A) in mammalian systems (B) in bacteria.

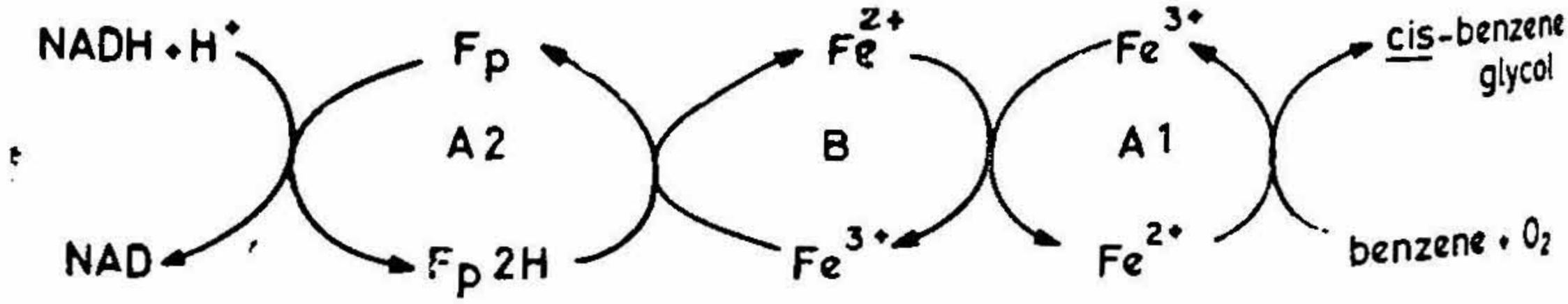


FIG. 2 (*b*). Suggested functions of components of benzene dioxygenase system.

Though the intermediate metabolite in the oxidation of benzene by bacteria was identified, the conversion could not be shown in a cell-free system for a long time. Washed cell suspensions of *Pseudomonas putida* were used to study benzene degradation and were shown to require NAD, Fe^{2+} and L-cysteine for maximum activity. It was initially suggested that benzene oxygenase is made up of at least two protein fractions[19]. Hogn and Jaenicke[22] showed that a cell-free extract of *Moraxella* is highly specific for the oxidation of benzene and requires catalytic amounts of NADH for this process.

This is regenerated during the course of the reaction by the dehydrogenation of *cis*-cyclohexadienediol to catechol. The dioxygenase was also shown to contain nonheme iron in the form of strongly bound Fe^{2+}.

Benzene oxygenase was obtained in a pure form from *Pseudomonas* grown on benzene as sole carbon source, by Axcell and Geary[23]. The enzyme system was shown to consist of three protein components and catalyzed the conversion of benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene. The three components of benzene oxygenase were designated as component A_1, A_2 and B. A_1 was shown to be an iron-sulfur protein with a pair of antiferromagnetically coupled Fe^{3+} atoms. Component A_2 was found to be a flavoprotein and B was shown to be a small, relatively stable nonheme iron protein. All the components were necessary for the enzyme activity and absence of any one of them resulted in total loss of activity. It was suggested that some organization of the components and cofactors is necessary for catalysis. The electrons from NADH are transferred to component A_2 with the concomitant reduction of bound FAD. The electrons are then transferred to component B and then to component A_1. These transfers are effected by Fe^{3+} to Fe^{2+} shuttle of the bound nonheme iron in the components B and A_1. The electrons are finally transferred to benzene which is converted to *cis*-benzeneglycol with the incorporation of both the atoms of molecular oxygen. The schematic representation for the organization of the components of benzene oxygenase is shown in Fig. 2. The next enzyme in the sequence, *cis*-benzeneglycol dehydrogenase has also been purified to homogeneity by Axcell and Geary[24]. The enzyme was shown to be specific for the *cis*-isomer of its substrate and required NAD^+ as hydrogen acceptor, Fe^{2+} and reduced glutathione for activity. The reaction catalyzed by dehydrogenase is shown in Fig. 2.

Double hydroxylation of naphthalene

The conversion of naphthalene to 1,2-dihydro-1,2-dihydroxynaphthalene and then to 1,2-dihydroxynaphthalene which undergoes ring cleavage, has been shown by several groups of workers, in different microorganisms[25–38]. It was known that during the mammalian oxidation of naphthalene, the hydrocarbon is first converted to an arene oxide by the cytochrome P–450 monooxygenase system[39–41]. Non-enzymatic isomerization to naphthols, reaction with cellular nucleophiles such as glutathione and enzymatic hydration to *trans*-1,2-dihydro-1,2-dihydroxynaphthalene are the principal reactions of the arene oxide. The dihydrodiol is further oxidized to a dihydroxy compound[39, 42]. A similar sequence of reactions was recently proposed by Cerniglia and Gibson[43] for the oxidation of naphthalene in *Cunninghamella elegans*. Various other fungi are also known to produce phenolic metabolites similar to those formed by hepatic microsomes[44, 45].

The uniform occurrence of *trans*-dihydrodiols as metabolites in mammals initially suggested that bacteria may also oxidize aromatic hydrocarbons through *trans*-dihydro-

diols. Earlier workers also reported that naphthalene and substituted naphthalenes were oxidized through *trans*-dihydrodiols[25, 46]. Contrary to the above suggestions, whole cells and cell-free extracts of *Pseudomonas* metabolized the *cis*-isomer of 1,2-dihydroxy-1,2-dihydronaphthalene at a much faster rate than the *trans*-isomer[47]. Radioactive trapping experiments with ^{14}C-naphthalene further confirmed the formation of *cis*-isomer in micro-organisms unlike the occurrence of *trans*-isomer in higher organisms[48]. Jerina *et al.*[48] using radioactive naphthalene resolved the isomer by thin layer chromatography and showed that several strains of bacteria produced only the *cis*-isomer. The *trans*-isomer, reported to be found in microorganisms by earlier workers was completely ruled out. Using a mutant *Pseudomonas*, the intermediate was isolated and identified as (+) *cis*-1 (R), 2 (S)-dihydro-1,2-dihydroxynaphthalene[50].

Naphthalene oxygenase, the first enzyme in the naphthalene pathway was reported to be coordinately induced with other enzymes of the pathway, namely, 1,2-dihydroxy-naphthalene oxygenase and salicylate dehydrogenase[51, 52]. Further studies by Williams *et al.*[53] showed that naphthalene and methylnaphthalene induce all the enzymes and the catechol formed is metabolized through the *meta* pathway. On the contrary, the latter compounds of the pathway, catechol and salicylic acid were found to induce the enzymes of the *ortho* pathway. Naphthalene-1,2-oxygenase activity was present in salicylate-grown cells. Thus, the inducer(s) of the early enzymes of the naphthalene degradation and of the *meta* pathway enzyme must be an early intermediate(s) of the pathway. Barnsley[54] found that salicylate induced all the enzymes of naphthalene metabolism and implicated salicylic acid in the induction of enzymes for naphthalene utilization. Recently, Dunn and Gunsalus[55] have provided evidence to believe that naphthalene utilization in *Pseudomonas* is through a plasmid. The organism was found to lose the capacity to utilize naphthalene spontaneously. The plasmid was also shown to code for naphthalene oxygenase.

Extracts of *Pseudomonas* grown on naphthalene was shown to oxidize a number of polycyclic aromatic hydrocarbon in the presence of NADH[56]. The ability to oxidize naphthalene was rapidly destroyed in air and on dialysis, but was restored by various reducing agents. Because of its instability, only a two-fold purification of naphthalene oxygenase was achieved. The enzyme required either NADPH or NADH and was shown to contain tightly bound Fe^{2+}.[49] In a recent communication, Laborde and Gibson[57] have reported that naphthalene oxygenase from *Pseudomonas* sp. is similar to the benzene oxygenase described earlier. However, detailed report on the enzyme is not available though it is known that it catalyzes the formation of *cis*-1,2-dihydro-1,2-dihydroxy-naphthalene from naphthalene. The *cis*-dihydrodiol was shown to be catalyzed by an NAD^+-dependent dehydrogenase, to 1,2-dihydroxynaphthalene. This enzyme was found to be specific for the *cis*-isomer of the dihydrodiol[49].

The scheme for the oxidation of naphthalene in bacteria and higher organisms is shown in Fig. 3.

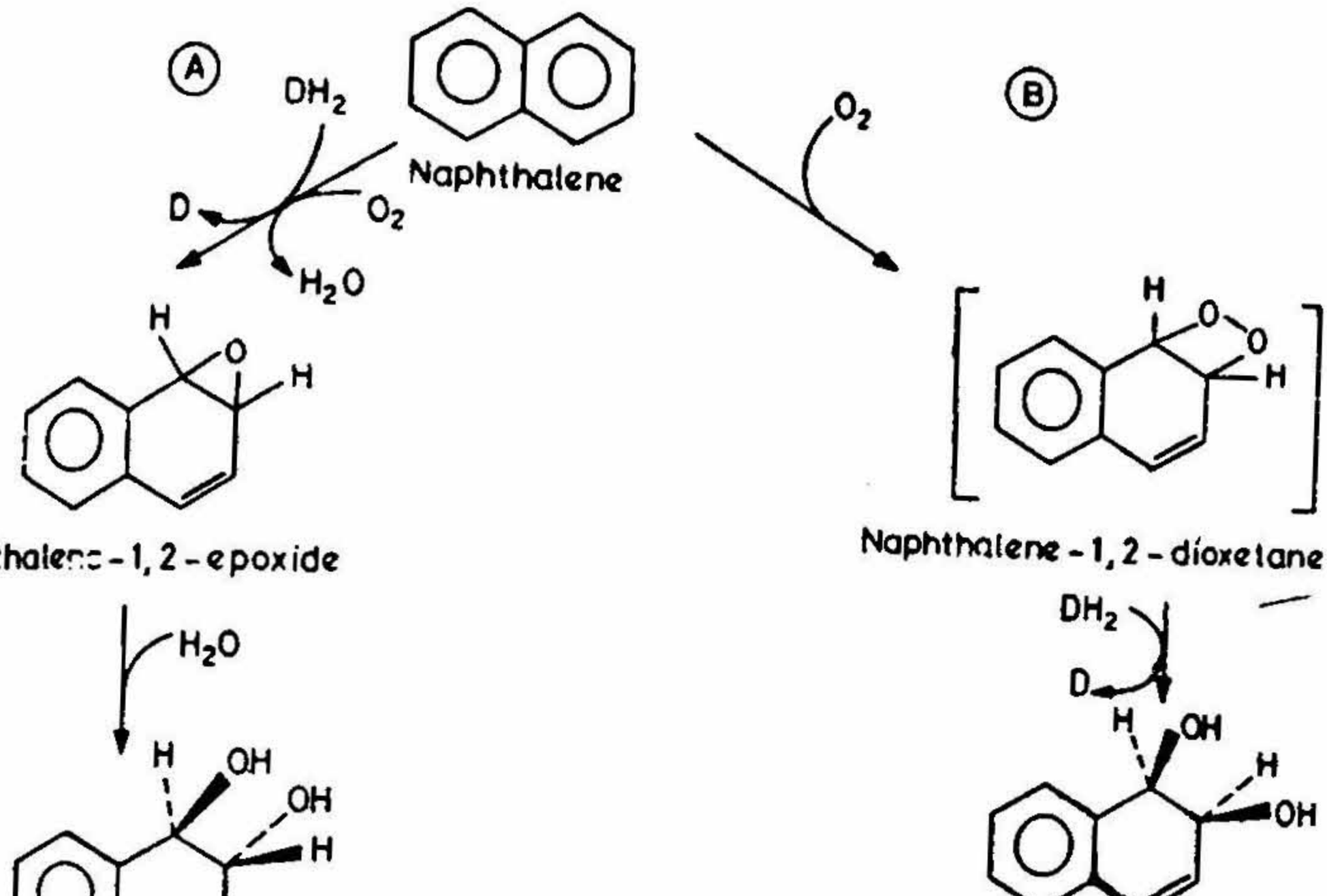


FIG. 3. Metabolism of naphthalene (A) in higher organisms (B) in bacteria.

Double hydroxylation of phenanthrene and anthracene

Earlier studies on the oxidation of phenanthrene by *Pseudomonas*, showed the formation of salicylic acid and 1-hydroxy-2-naphthoic acid as intermediates[58, 59]. Colla *et al.*[60] and Kaneko *et al.*[62] independently identified 3,4-dihydro-3,4-dihydroxy phenanthrene from cultures of *Flavobacterium* metabolizing phenanthrene. Dihydroxyphenanthrene was also proposed by Kiyohara *et al.*[62] as an intermediate in the oxidation of phenanthrene by *Aeromonas*. Evans and his collaborators[63] made an initial observation that phenanthrene is oxidatively metabolized by soil *Pseudomonas* through *trans*-3,4-dihydro-3,4-dihydroxyphenanthrene to 3,4-dihydroxyphenanthrene. Anthracene was similarly shown to be converted to *trans*-1,2-dihydro-1,2-dihydroxyanthracene and then to 1,2-dihydroxyanthracene.

The absolute stereochemistry of the dihydrodiol produced from anthracene by mammals and bacteria was assigned by Akthar *et al.*[64] The *trans*-1,2-dihydro-1,2-dihydroxyanthracene and *cis*-1,2-dihydro-1,2-dihydroxyanthracene were isolated as major metabolites of anthracene from rabbits and *Beijerinckia* respectively. The *cis* and *trans* configuration of the dihydrodiols produced from anthracene and phenanthrene by bacterial and mammalian systems respectively, was also shown by Jerina *et al.*[65] From mutant strains of *Beijerinckia* and *Pseudomonas* which were deficient in dihydrodiol dehydrogenases, they isolated *cis*-3,4-dihydro-3,4-dihydroxyphenanthrene and *cis*-1,2-dihydro-1,2-dihydroxyanthracene. The *cis*-1,2-dihydro-1,2-dihydroxyphenanthrene was also detected as a minor product from phenanthrene. The formation of *trans*-dihydrodiols in mammalian systems was shown by the isolation of epoxide hydrase which converts corresponding arene oxide to *trans*-dihydrodiol[65].

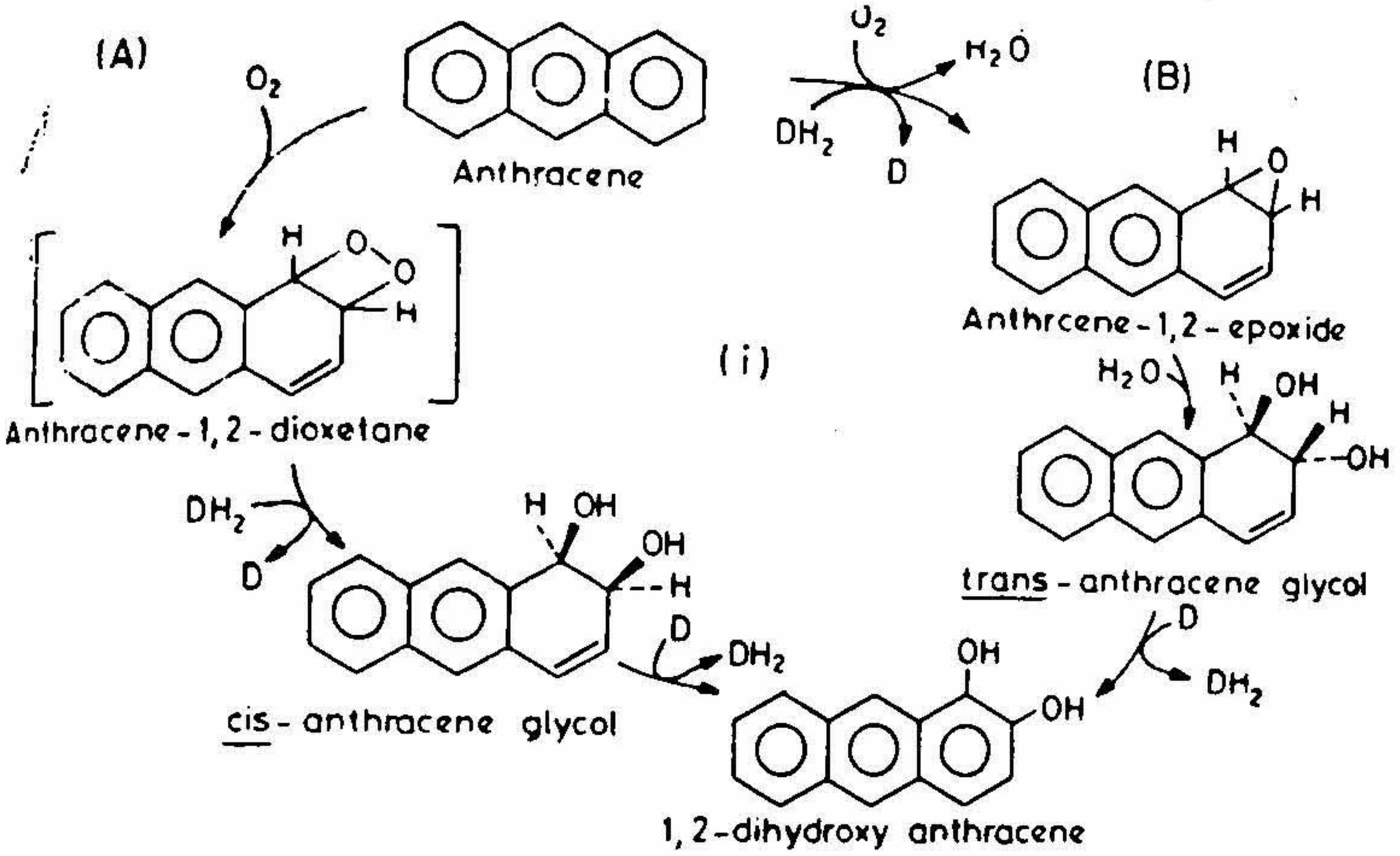
Rodgoff[66] observed that substitution of phenanthrene by a methyl group at '9' position blocks the oxidation of the compound by a resting cell suspension of a phenanthrene-grown soil *Pseudomonas*. When 2-methylphenanthrene was provided, the oxidation rate was observed to be considerably higher. 3-methylphenanthrene is oxidized at an intermediate rate between the above two, even though the methyl group is attached to 'C' directly involved in ring splitting.

These data are in agreement with the hypothesis that polynuclear aromatic hydrocarbons attach to the relevant bacterial enzymes at C–C bonds of high electron density and that ring splitting reactions then occur at other bonds on the substituted molecule. The actual bond that undergoes fission is determined by the electronic and steric configurations of the enzyme-substrate complex. When linearly arranged aromatic compounds such as naphthalene or anthracene are attacked, attachment to the enzyme and ring splitting may take place on the same ring. On the other hand, angular aromatic compounds such as phenanthrene afford attachment to the enzyme at a bond in a region other than the one containing the ring splitting site[66].

Thus the metabolic formation of vicinal dihydrodiols is one of the principal pathways by which both mammals and bacteria oxidize phenanthrene and anthracene. The dihydrodiols of mammalian origin have *trans*-configuration and result from enzymic hydration of arene oxides[61,64,67]. The arene oxides are formed by monooxygenation of the aromatic nucleus. The dihydrodiols of bacterial origin have *cis*-configuration and result from the action of dioxygenases. The dioxygenases that convert anthracene and phenanthrene to their respective dihydrodiols have not been isolated so far. However, the reaction they catalyze is clear from the intermediates isolated. The schematic representation of the degradation of anthracene and phenanthrene by bacterial and mammalian systems is shown in Fig. 4.

Double hydroxylation of toluene and xylene

Two bacterial strains, *Pseudomonas* and *Achromobacter* were shown to grow on toluene as sole carbon source. The toluene-grown cells oxidized 3-methylcatechol without any



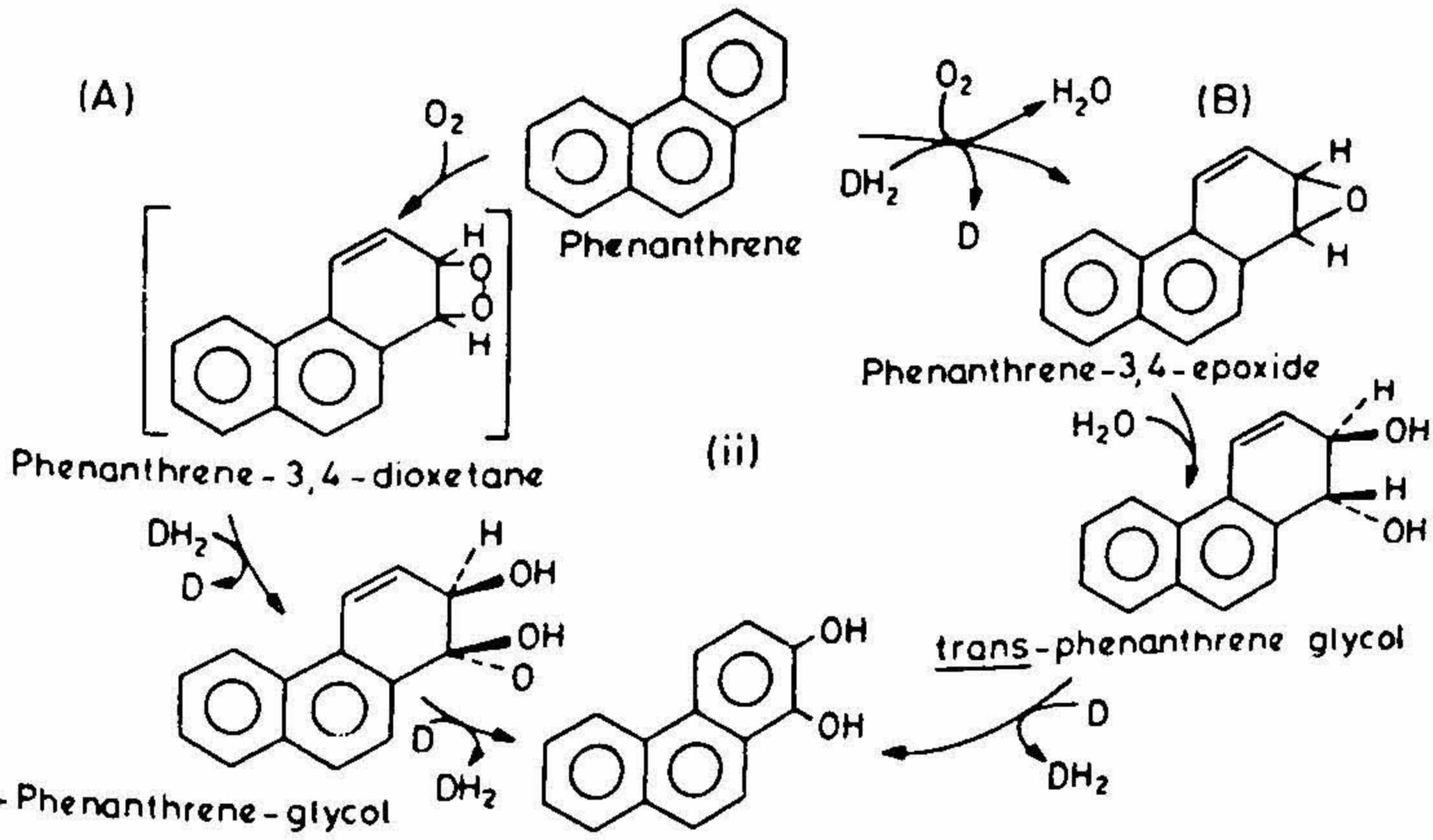


FIG. 4. Oxidation of (i) anthracene and (ii) phenanthrene by (A) bacteria and (B) mammals.

lag period[68]. 3-methylcatechol was also detected in toluene-oxidizing cultures in the early log phase. From the culture medium of a mutant *Pseudomonas*, a neutral compound was isolated by Karplus[69] and identified as 2,3-dihydro-2,3-dihydroxytoluene.

But the stereochemistry of the hydroxyl groups were not established by them. Gibson *et al.*[70] isolated two compounds from a mutant *Pseudomonas* growing on 4-chlorctoluene. These compounds were identified as *cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene and 4-chloro-2,3-dihydroxy-1-methyl benzene. *cis*-2,3-dihydroxy-1-methyl-cyclohexa-4,6-diene (*cis*-toluneglyco!) was also isolated from a mutant *Pseudomonas* growing on toluene[71, 72]. The formation of *cis*-toluneglycol was further confirmed when it was shown to be oxidized to 3-methylcatechol by whole cells[69, 70] and cell-free extracts of *Pseudomonas*[71, 73].

The conversion of toluene to *cis*-toluneglycol is effected by toluene dioxygenase (Fig. 5). Nozaka and Kusunose[74, 75] reported the requirement for three protein fractions for toluene hydroxylation by *Pseudomonas aeruginosa*. A crude extract of the organism was fractionated into three protein components on DEAE-cellulose. With NADH and FAD as cofactors, all the three protein fractions were required for maximum activity. On ammonium sulfate fractionation, two protein components were resolved which had

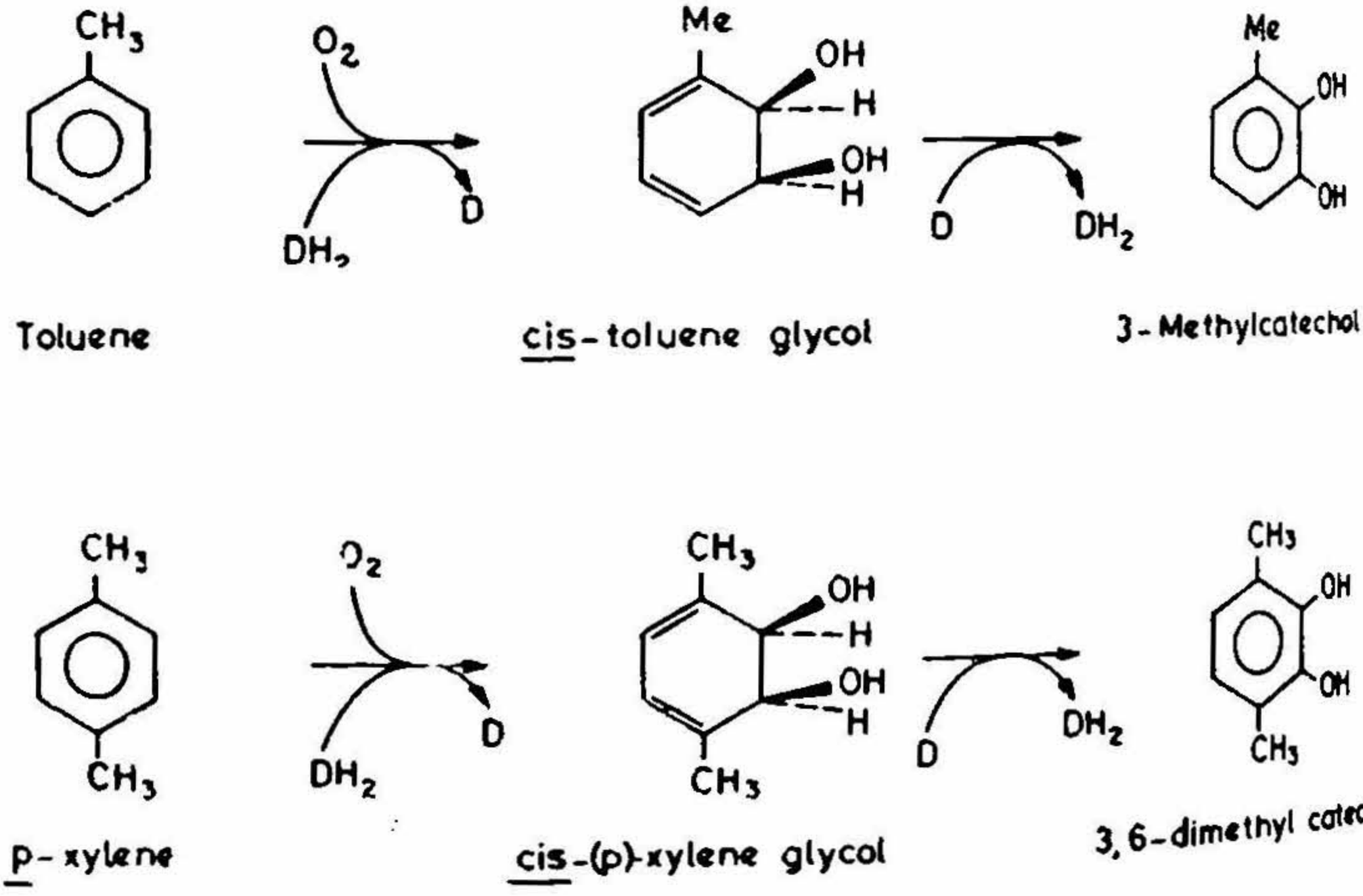


FIG. 5. Scheme for the oxidation of (A) toluene (B) *p*-xylene (C) *m*-xylene.

to be combined for maximum activity. Recently, the multienzyme system, toluene dioxygenase. was purified by Yeh *et al.*[76] and shown to be made up of three protein components. The yellow protein A_1 plays an important role in transfer of electrons to cytochrome *c*. The hydroxylation is stimulated by the presence of the brown protein, B, which has two moles of iron and two moles of inorganic sulfur per mole of protein. The organization of the enzyme is apparently similar to the benzene oxygenase system described earlier. The oxidation of toluene is shown in Fig. 5. The next enzyme in the pathway, *viz.*, *cis*-toluenedihydrodiol dehydrogenase has been purified to homogeneity by Rogers and Gibson[77]. The enzyme is specific for the *cis*-isomer.

The utilization of xylene by *Nocardia corralinas* under co-oxidation conditions was reported by Jamison *et al*[78–80]. *a,a'*-dimethyl-*cis,cis*-muçonic acid was found to be the accumulated intermediate. Two pathways were proposed for the degradation of *p*-xylene.

(*a*) A dioxygenation and formation of 2,3-dihydroxy *p*-xylene as intermediate.

(*b*) Degradation of *p*-xylene to *p*-toluic acid and dioxygenation of *p*-toluic acid (This is discussed separately under dihydroxylation of aromatic acids).

Gibson *et al.*[81] isolated an accumulated compound from the culture filtrates of a mutant *Pseudomonas* growing on *p*-xylene as sole carbon source. Similarly another accumulated compound was isolated during the oxidation of *m*-xylene by the organism. These two compounds were identified as *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (*cis-p*-xylenediol) and *cis*-3,5-dimethyl-3,5-cyclohexadiene-1,2-diol. Both the reactions are catalyzed by dioxygenase. It is not clear whether there are separate dioxygenases for the oxidation of *p*- and *m*-xylene or the same dioxygenase catalyzes the oxidation of both substrates. The scheme for the oxidation of *p*- and *m*-xylene is shown in Fig. 5.

Double hydroxylation of biphenyl

The conversion of biphenyl to 2,3-dihydroxybiphenyl in bacterial systems was shown by a number of workers. Lunt and Evans[82] isolated 2,3-dihydroxybiphenyl from pure cultures of gram negative soil bacteria utilizing biphenyl. Catelani *et al.*[83] showed that cultures of *Pseudomonas putida* grown on biphenyl yielded 2,3-dihydro-2,3-dihydroxybiphenyl. By incubation of washed suspensions of *Pseudomonas* with 2,3-dihydroxybiphenyl, further metabolites were isolated. Ring fission was shown to occur between C_1 and C_2 of 2,3-dihydroxybiphenyl[84, 85]. The above compounds were also proposed as intermediates in the degradation of biphenyl and *p*-chlorobiphenyl by *Achromobacter*[86]. However, it was not clear from the above studies whether the dihydro dihydroxy intermediate has a *cis* or *trans* configuration.

Gibson *et al.*[87] isolated a species of *Beijerinckia* that utilizes biphenyl as sole carbon source for growth. *cis*-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene was isolated and

identified from a mutant *Beijerinckia*[88]. Cell-free extracts prepared from the para organisms oxidized *cis*-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene to 2,3-dihydro biphenyl[87]. Hence a *trans*-dihydrodiol intermediate was ruled out.

The scheme for the microbial degradation of biphenyl is shown in Fig. 6. The in step is apparently catalyzed by a dioxygenase which has not been shown in cell-free syste

Double hydroxylation of benzo (a) pyrene and benzo(a) anthracene

Gibson and his group[89, 90] isolated a mutant strain of *Beijerinckia* which oxidize benzo(a) pyrene and benzo(a) anthracene, when grown on succinate in the presence biphenyl. The organism was shown to contain an enzyme system that oxidized benzo(pyrene and benzo(a) anthracene to a mixture of vicinal dihydrodiols. The major dihydro diol formed from benzo(a) pyrene was identified as *cis*-9,10-dihydroxy-9,10-dihydr benzopyrene by comparison with a synthetic sample (Fig. 7). Benzo(a) anthracene wa metabolized to four dihydrodiols, the major isomer being *cis*-1,2-dihydro-1,2-dihydro benzo (a) anthracene (Fig. 7). On the contrary, higher organisms oxidize the abo compounds tc *trans*-vicinal dihydrodiols through arene oxide intermediates[41, 91].

From the available data, it can be generalized that the initial reaction in the bacteria oxidation of aromatic hydrocarbons involves the formation of a *cis*-dihydrodiol probably *via* a cyclic peroxide type intermediate. In contrast, the eukaryotic organisms oxidize aromatic hydrocarbons to arene oxides which are hydrated to form *trans*-dihydro diol. It is conceivable that while the prokaryotic organisms employ a dioxygenase to initiate the metabolism of aromatic hydrocarbons, eukaryotic organisms employ a mono oxygenase enzyme system. The generalization is summarized in Fig. 8.

3. Double hydroxylation of indole

Sakamoto *et al.*[92] who studied the decomposition of indole in a tapwater bacterium, detected the following compounds in the culture filtrate. Isatin, formylanthranilic acid, anthranilic acid, salicylic acid and catechol. Based on these findings they proposed the following pathway for the degradation of indole. Indole → Indoxyl → dihydroxy-indole → Isatin → formylanthranilic acid → anthranilic acid → catechol. Fujioka and Wada[93] isolated a soil microorganism which utilized indole as sole source of carbon and nitrogen. Dihydroxyindole was detected as the intermediate in the metabolism of indole. Direct evidence for the formation of dihydroxyindole as intermediate was not obtained since indole was oxidized to anthranilic acid without the accumulation of dihydroxyindole. However, when skatole was incubated with indole-grown cells, the compound was oxidized with the consumption of one mole of oxygen per mole of the substrate to (+)-2-oxo-3-methyl-3-hydroxyindoline. This compound was not further metabolized in the organism. Skatole does not have a β hydrogen atom and hence enolization of the intermediate formed is not possible. Therefore, ketol accumulates

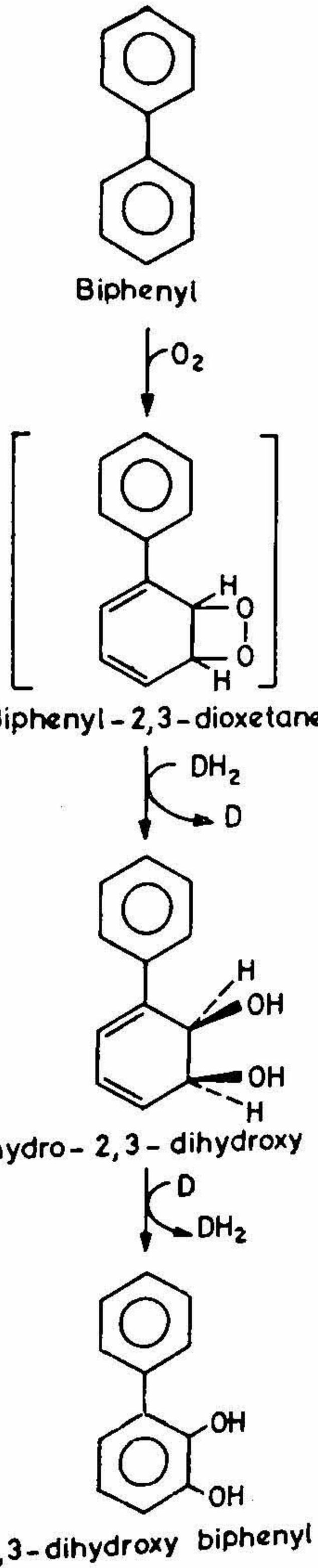


FIG. 6. Bacterial degradation of biphenyl.

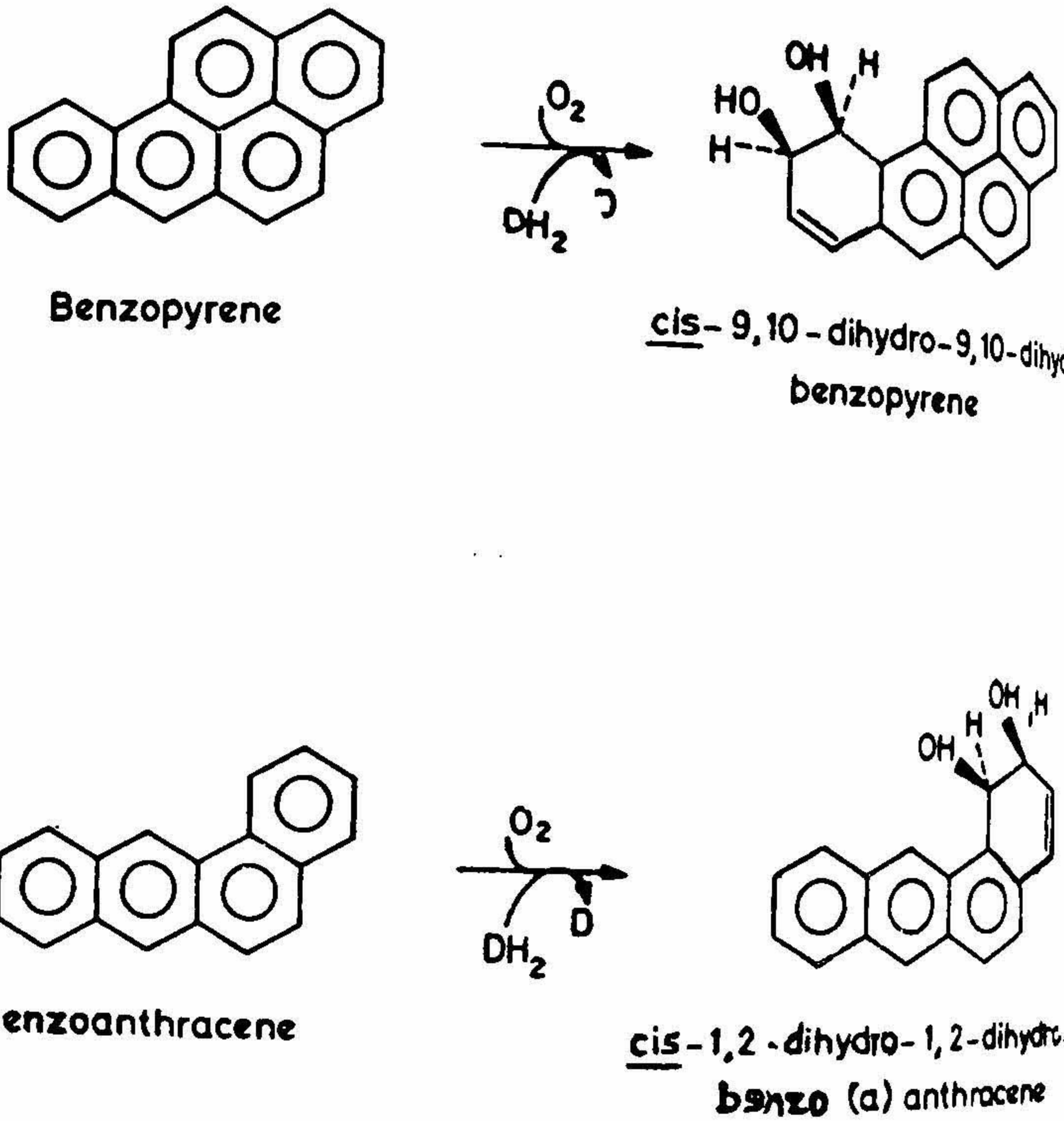


FIG. 7. Oxidation of benzopyrene and benzoanthracene by microorganism.

in the medium. The formation of 2-oxo-3-methyl-3-hydroxyindoline from skatole as the induction of dihydroxyindole oxygenase by indole strongly suggests that indole is metabolized to anthranilic acid *via* dihydroxyindole. Indole oxygenase was also partially purified from the soil microorganism.

It is interesting to note that in the case of indole, no dihydrodiol intermediate is formed as in the case of other aromatic hydrocarbons discussed earlier.

The enzyme catalyzing the oxidation of indole to dihydroxyindole could not be solubilized. The activity was found to be associated with the cellular debris. The oxidation of indole is shown in Fig. 9. Though an epoxide mechanism has been proposed by the authors, a cyclic peroxide intermediate is more likely as the formation of 2-oxo-3-methyl-3-hydroxyindole from skatole could be better explained with the cyclic peroxide intermediate rather than the epoxide intermediate.

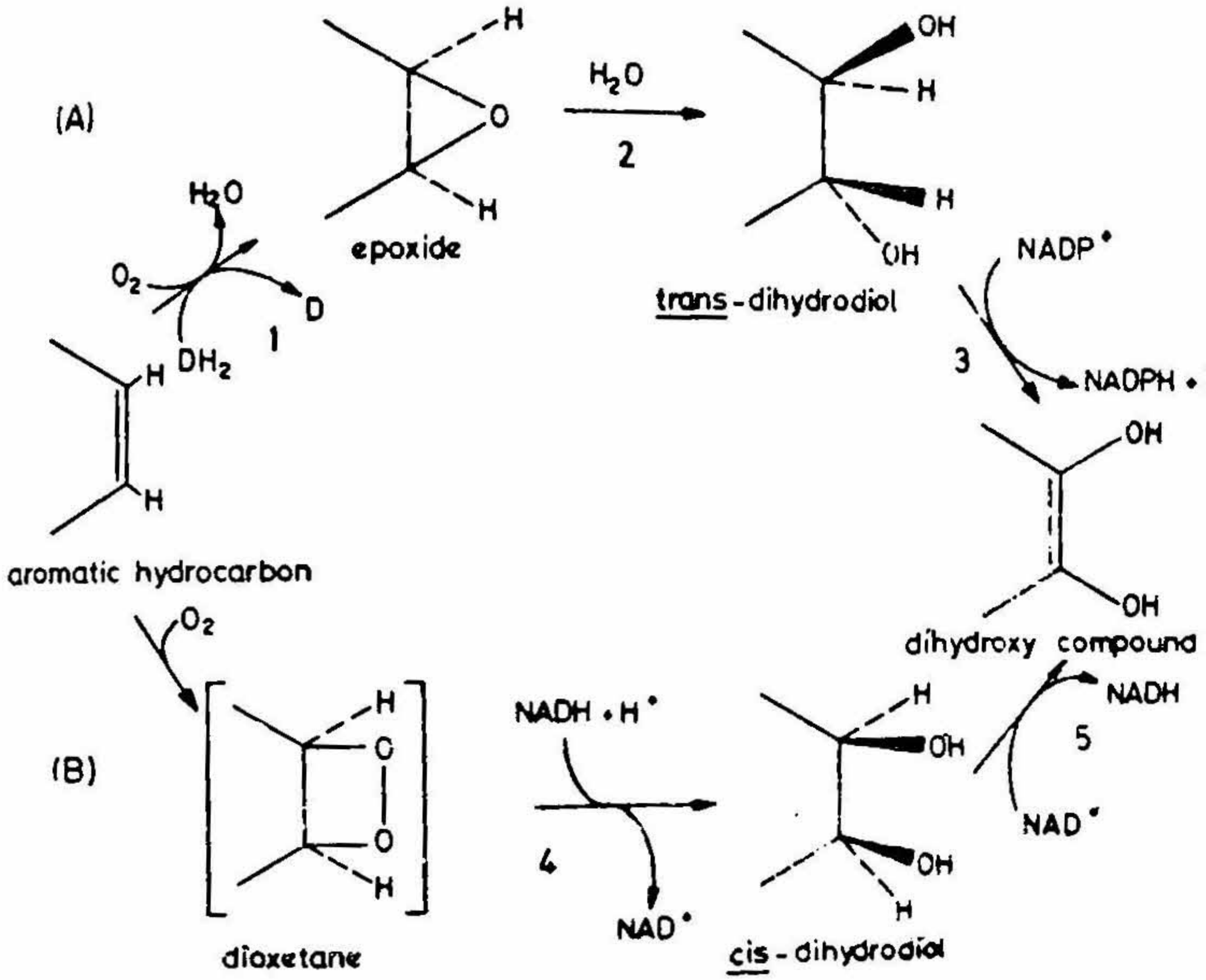


FIG. 8. Initial reactions in the oxidation of aromatic hydrocarbons by (A) higher organisms (B) bacteria.

1. Aromatic hydrocarbon monooxygenase
2. Epoxide hydrase
3. *trans*-dihydrodiol dehydrogenase
4. Aromatic hydrocarbon dioxygenase
5. *cis*-dihydrodiol dehydrogenase

4. Double hydroxylation of pyrazon

Pyrazon, an active ingredient in the herbicide pyramin, was found to be utilized by soil bacteria as sole carbon source. But the metabolites were not identified[94]. DeFrenne *et al.*[95] during their studies on pyrazon degradation by an unidentified bacterium, isolated four metabolites from the culture filtrates. They were identified as:

(*a*) 5-amino-4-chloro-2-(2,3-*cis*-dihydroxycyclohexa-4,6-diene-1-yl)-3(2H)pyridazinone.

(*b*) 2-(5-amino-4-chloro-3-oxo-2,3-dihydro-2-pyridazino)-*cis*, *cis*-muconic acid.

(*c*) 2-pyrone-6-carboxylic acid and

(*d*) 5-amino-4-chloro-3(2H)pyridazinone.

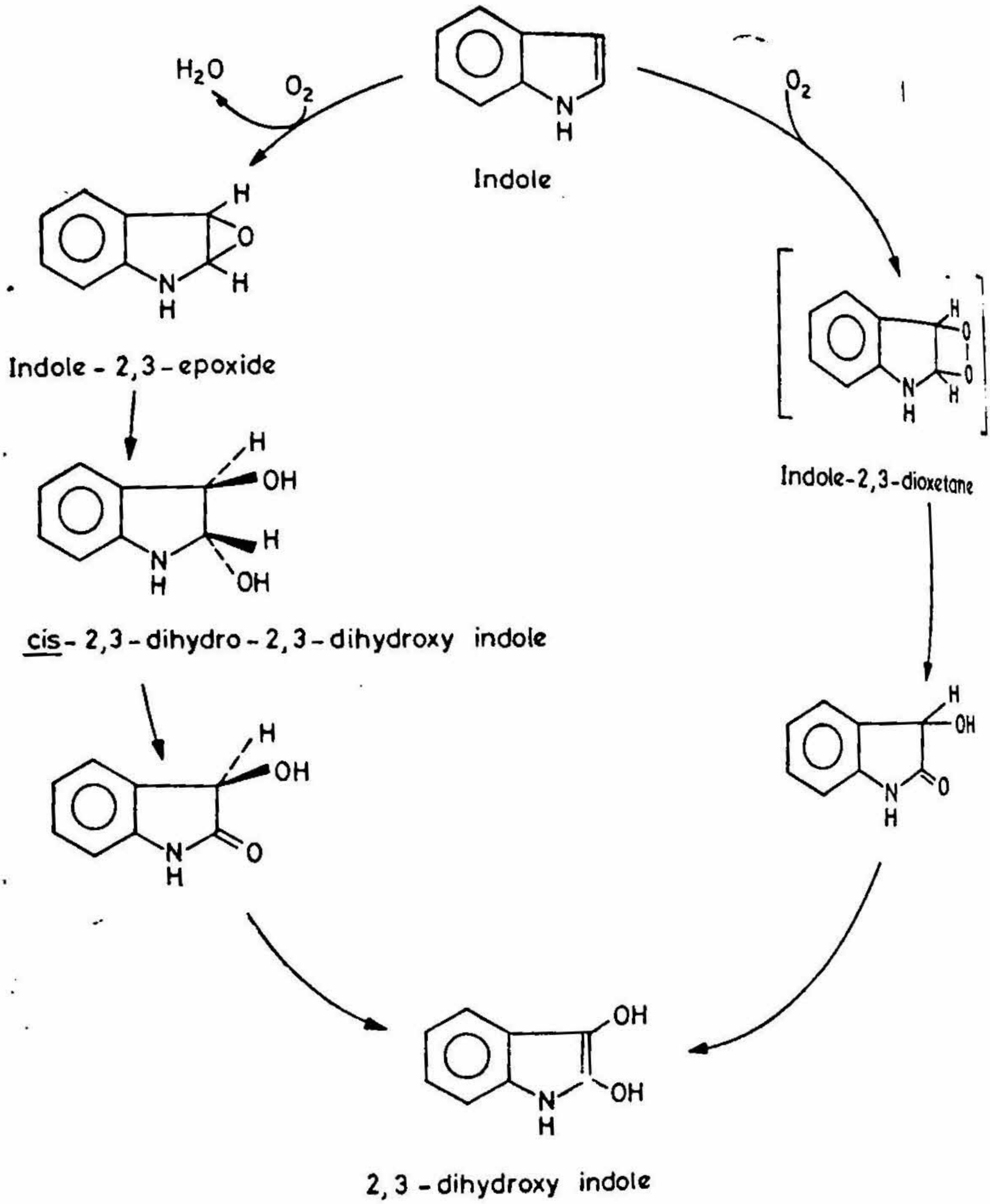


FIG. 9. Oxidation of indole by bacteria.

Compound (*a*) was found to be formed in stoichiometric amounts from pyrazon. The pathway proposed for pyrazon degradation is shown in Fig. 10(*a*). Subsequent studies carried out by Lingen's group[96], led to the isolation of 5-amino-4-chloro-2-(2,3-dihydroxyphen-1-yl)-3(2H)pyridazinone. The compound was also prepared by enzymatic and chemicai treatment of 5-amino-4-chloro-2-(2,3-dihydroxycyclohexa-4,6-diene-1-yl) pyridazinone.

worked out by several groups of investigators using various biological materials, namely, plants, animals as well as microorganisms. The map includes not only many degradative processes, but also biosynthetic pathways of physiologically important compounds. It is apparent that most of the oxidative steps are catalyzed by oxygenases rather than by oxidases or dehydrogenases.

Oxygenases are classified as shown in Table I

Table I

Classification of oxygenases

Oxygenase

A. *Monooxygenases*

1. Internal monooxygenase

2. External monooxygenase
   - (*a*) Pyridine nucleotide-linked flavoprotein
   - (*b*) Flavin-linked hemoproteins
   - (*c*) Iron sulfur protein-linked hemoprotein
   - (*d*) Pteridine-linked monooxygenase
   - (*e*) Ascorbate-linked copper proteins
   - (*f*) With another substrate as reductant

B. *Dioxygenase*

1. Intramolecular dioxygenase
   - (*a*) Hemoproteins
   - (*b*) Nonheme iron protein
   - (*c*) Copper protein
   - (*d*) Flavoprotein

2. Intermolecular dioxygenase

3. Miscellaneous

Cleavage of the benzene nucleus has always been thought of as the most important function of dioxygenases. However, recent studies on the metabolism of a number of compounds in microorganisms have shown the existence of another class of dioxygenases which carry out the double hydroxylation of the benzene ring, thus preparing an aromatic nucleus for attack by ring fission dioxygenases. The compounds which undergo dihydro-

xylation include hydrocarbons like benzene, naphthalene, anthracene, pyrene, phenanthrene, benzopyrene, toluene, xylene, biphenyl, aromatic acids like benzoic acid, phthalic acid, toluic acid, cumic acid, and aromatic amines like aniline, anthranilic acid and the recently studied herbicide, pyrazon.

Some of the double hydroxylating enzymes from bacteria, like anthranilate hydroxylase and benzoate oxygenase were discovered much earlier[1,2]. These enzymes have been classified under 'Miscellaneous' as shown in Table I. However, with the recent discovery of new enzyme systems catalyzing similar dihydroxylation reactions this class of dioxygenases requires to be separately classified. The classification of dioxygenases, shown in Table I can be suitably altered to include the dihydroxylating dioxygenases, as follows :

Dioxygenases

- Intermolecular
- Intramolecular
  - Ring cleaving
    - *a.* Hemoproteins
    - *b.* Nonheme iron proteins
    - *c.* Copper proteins
    - *d.* Flavoproteins
  - Ring hydroxylating
    - Ex : Anthranilate hydroxylase (bacteria)

Excellent review articles on various aspects of oxygenases that present a detailed account of their catalytic and biological functions are available[2-10]. In the following pages, discussion will be focussed on the recent developments in the area of ring hydroxylating dioxygenases from microorganisms.

2. Dihydroxylation of aromatic hydrocarbons

Aromatic hydrocarbons are found in nature as constituents of fossil fuels. The aromatic fraction generally constitutes less than 20% of total composition of most crude oil. Though it has been known for many years, that certain aromatic hydrocarbons are substrates for bacterial oxidation, the number of different structures known to be subject to microbial degradation represent a very small percentage of the aromatic hydrocarbons. Ranging from benzene to the polynuclear benzopyrene, the utilization of aromatic hydrocarbons by microorganisms has received much attention during the last decade. It has emerged that the degradation of aromatic hydrocarbons is initiated by a mechanism that differs from that of any monooxygenase reaction known to date, in so far as both the

atoms of molecular oxygen are incorporated simultaneously into the substrate as two hydroxyl groups[11, 12]. It appears that one molecule of oxygen is added to the aromatic ring to form a dioxetane intermediate and that the NADH required in the dihydroxylation is utilized for reducing the dioxetane to a dihydrodiol. Very few enzyme systems that catalyze the initial oxidation reaction mentioned above, have been isolated and studied. However, a number of intermediates isolated in recent years clearly illustrate the involvement of such enzyme systems in the oxidation of a number of aromatic compounds. In the following pages, the dihydroxylation reactions of aromatic hydrocarbons will be discussed.

Dihydroxylation of benzene

The bacterial oxidation of benzene appears to be confined to those species which are capable of forming the enzymes of the so-called aromatic pathway. The first step in the oxidation of benzene and other compounds is apparently unique and can be carried out only by a limited number of species. In the case of benzene, the enzymes for its degradation are inducible. Two organisms, a strain of *Pseudomonas* and *Mycobacterium* species capable of utilizing benzene as sole carbon source were isolated from soil by enrichment culture technique[13].

For many years, it was assumed that benzene is oxidized by bacteria through phenol. Park and William[12] showed the conversion of benzene to catechol without the intermediate formation of phenol. Haccius and his group[14, 15] isolated an organism capable of oxidizing benzene to catechol and identified it as *Nocardia corallina*. Nakajuna and Toida[16] and Mair and Stone[13] proposed a hypothesis that dihydrodiol is an intermediate in benzene degradation.

Gibson and his associates[17, 18] proposed a pathway for the degradation of benzene which involved a hypothetical cyclic peroxide intermediate. In mammalian system, epoxide is the proposed intermediate and *trans*-1,2-dihydro-1,2-dihydroxybenzene, the identified metabolite[19]. The scheme for the oxidation of benzene by bacterial and mammalian system is shown in Fig. 2.

Further Gibson *et al.*[20,21] showed that appropriately induced cultures of *Pseudomonas putida* oxidize benzene and ethylbenzene at equal rates. A mutant strain of this organism when grown on benzene accumulated *cis*-1,2-dihydroxycyclohexa-3,5-diene in the medium. The product was found to be identical with a synthetic sample of *cis*-1,2-dihydroxycyclohexa-3,5-diene. Experiments with isotopic oxygen showed the incorporation of both the atoms of molecular oxygen into *cis*-1,2-dihydroxycyclohexa-3,5-diene. The same compound was also isolated from a benzene utilizing *Moraxella*, B, by Hogn and Jaenicke[22]. Both the atoms of oxygen were shown to be incorporated into the above compound, simultaneously. The above results suggest that the initial reaction in benzene oxidation by *Pseudomonas* and *Moraxella*, involves a dioxygenation.

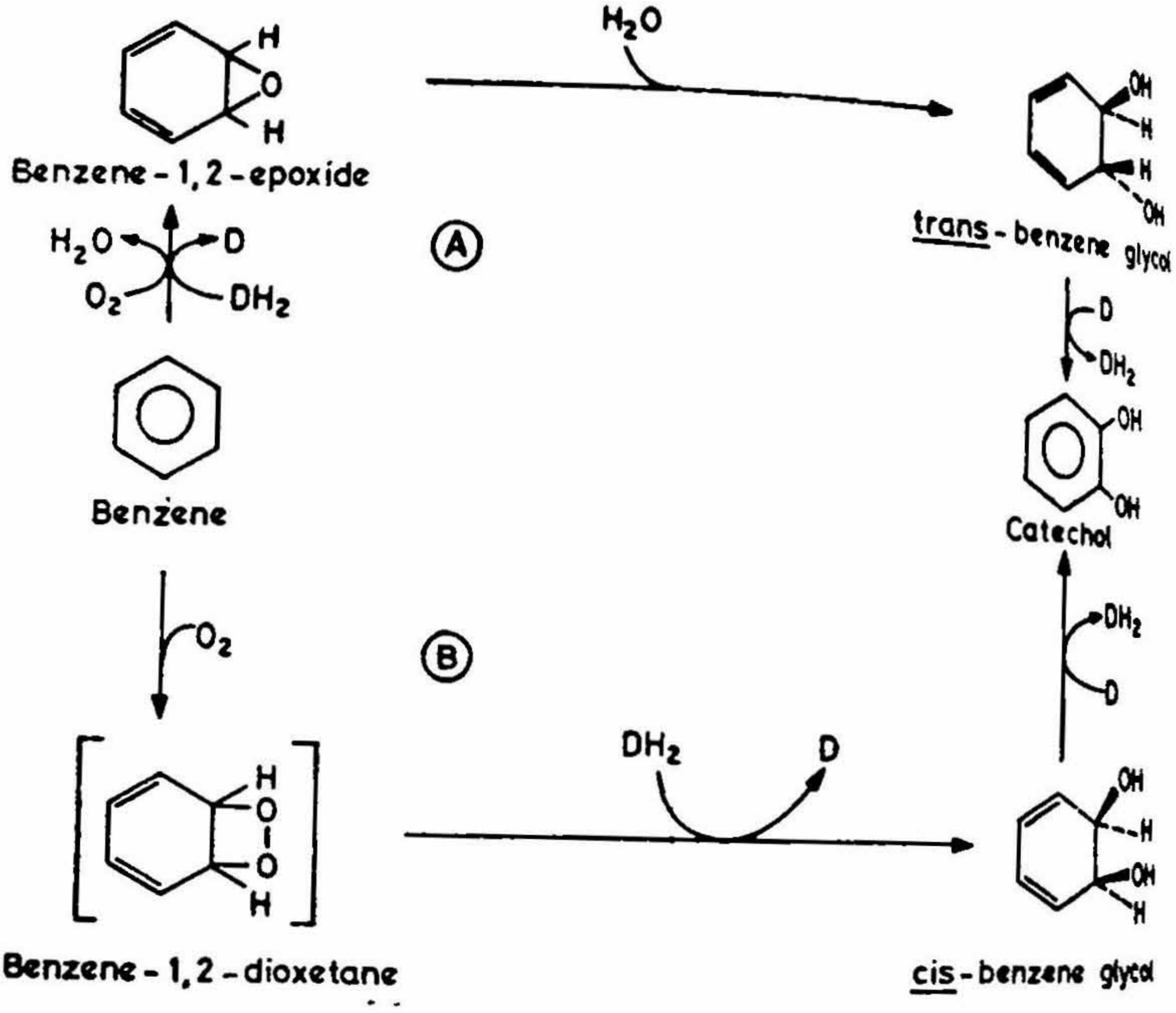


FIG. 2 (*a*). Metabolism of benzene (A) in mammalian systems (B) in bacteria.

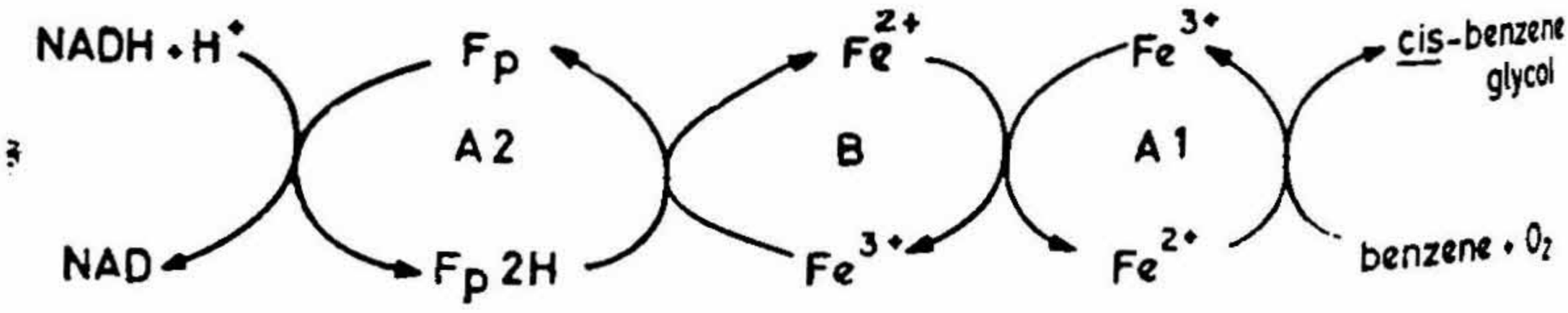


FIG. 2 (*b*). Suggested functions of components of benzene dioxygenase system.

Though the intermediate metabolite in the oxidation of benzene by bacteria was identified, the conversion could not be shown in a cell-free system for a long time. Washed cell suspensions of *Pseudomonas putida* were used to study benzene degradation and were shown to require NAD, Fe^{2+} and L-cysteine for maximum activity. It was initially suggested that benzene oxygenase is made up of at least two protein fractions[19]. Hogn and Jaenicke[22] showed that a cell-free extract of *Moraxella* is highly specific for the oxidation of benzene and requires catalytic amounts of NADH for this process.

This is regenerated during the course of the reaction by the dehydrogenation of *cis*-cyclohexadienediol to catechol. The dioxygenase was also shown to contain nonheme iron in the form of strongly bound Fe^{2+}.

Benzene oxygenase was obtained in a pure form from *Pseudomonas* grown on benzene as sole carbon source, by Axcell and Geary[23]. The enzyme system was shown to consist of three protein components and catalyzed the conversion of benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene. The three components of benzene oxygenase were designated as component A_1, A_2 and B. A_1 was shown to be an iron-sulfur protein with a pair of antiferromagnetically coupled Fe^{3+} atoms. Component A_2 was found to be a flavoprotein and B was shown to be a small, relatively stable nonheme iron protein. All the components were necessary for the enzyme activity and absence of any one of them resulted in total loss of activity. It was suggested that some organization of the components and cofactors is necessary for catalysis. The electrons from NADH are transferred to component A_2 with the concomitant reduction of bound FAD. The electrons are then transferred to component B and then to component A_1. These transfers are effected by Fe^{3+} to Fe^{2+} shuttle of the bound nonheme iron in the components B and A_1. The electrons are finally transferred to benzene which is converted to *cis*-benzeneglycol with the incorporation of both the atoms of molecular oxygen. The schematic representation for the organization of the components of benzene oxygenase is shown in Fig. 2. The next enzyme in the sequence, *cis*-benzeneglycol dehydrogenase has also been purified to homogeneity by Axcell and Geary[24]. The enzyme was shown to be specific for the *cis*-isomer of its substrate and required NAD^+ as hydrogen acceptor, Fe^{2+} and reduced glutathione for activity. The reaction catalyzed by dehydrogenase is shown in Fig. 2.

Double hydroxylation of naphthalene

The conversion of naphthalene to 1,2-dihydro-1,2-dihydroxynaphthalene and then to 1,2-dihydroxynaphthalene which undergoes ring cleavage, has been shown by several groups of workers, in different microorganisms[25–38]. It was known that during the mammalian oxidation of naphthalene, the hydrocarbon is first converted to an arene oxide by the cytochrome P–450 monooxygenase system[39–41]. Non-enzymatic isomerization to naphthols, reaction with cellular nucleophiles such as glutathione and enzymatic hydration to *trans*-1,2-dihydro-1,2-dihydroxynaphthalene are the principal reactions of the arene oxide. The dihydrodiol is further oxidized to a dihydroxy compound[39, 42]. A similar sequence of reactions was recently proposed by Cerniglia and Gibson[43] for the oxidation of naphthalene in *Cunninghamella elegans*. Various other fungi are also known to produce phenolic metabolites similar to those formed by hepatic microsomes[44, 45].

The uniform occurrence of *trans*-dihydrodiols as metabolites in mammals initially suggested that bacteria may also oxidize aromatic hydrocarbons through *trans*-dihydro-

diols. Earlier workers also reported that naphthalene and substituted naphthalenes were oxidized through *trans*-dihydrodiols[25, 46]. Contrary to the above suggestions, whole cells and cell-free extracts of *Pseudomonas* metabolized the *cis*-isomer of 1,2-dihydroxy-1,2-dihydronaphthalene at a much faster rate than the *trans*-isomer[47]. Radioactive trapping experiments with ^{14}C-naphthalene further confirmed the formation of *cis*-isomer in micro-organisms unlike the occurrence of *trans*-isomer in higher organisms[48]. Jerina *et al.* using radioactive naphthalene resolved the isomer by thin layer chromatography and showed that several strains of bacteria produced only the *cis*-isomer. The *trans*-isomer, reported to be found in microorganisms by earlier workers was completely ruled out. Using a mutant *Pseudomonas*, the intermediate was isolated and identified as (+) cis-1 (R), 2 (S)-dihydro-1,2-dihydroxynaphthalene[50].

Naphthalene oxygenase, the first enzyme in the naphthalene pathway was reported to be coordinately induced with other enzymes of the pathway, namely, 1,2-dihydroxy-naphthalene oxygenase and salicylate dehydrogenase[51, 52]. Further studies by Williams *et al.*[53] showed that naphthalene and methylnaphthalene induce all the enzymes and the catechol formed is metabolized through the *meta* pathway. On the contrary, the latter compounds of the pathway, catechol and salicylic acid were found to induce the enzymes of the *ortho* pathway. Naphthalene-1,2-oxygenase activity was present in salicylate-grown cells. Thus, the inducer(s) of the early enzymes of the naphthalene degradation and of the *meta* pathway enzyme must be an early intermediate(s) of the pathway. Barnsley[54] found that salicylate induced all the enzymes of naphthalene metabolism and implicated salicylic acid in the induction of enzymes for naphthalene utilization. Recently, Dunn and Gunsalus[55] have provided evidence to believe that naphthalene utilization in *Pseudomonas* is through a plasmid. The organism was found to lose the capacity to utilize naphthalene spontaneously. The plasmid was also shown to code for naphthalene oxygenase.

Extracts of *Pseudomonas* grown on naphthalene was shown to oxidize a number of polycyclic aromatic hydrocarbon in the presence of NADH[56]. The ability to oxidize naphthalene was rapidly destroyed in air and on dialysis, but was restored by various reducing agents. Because of its instability, only a two-fold purification of naphthalene oxygenase was achieved. The enzyme required either NADPH or NADH and was shown to contain tightly bound Fe^{2+}.[49] In a recent communication, Laborde and Gibson[5] have reported that naphthalene oxygenase from *Pseudomonas* sp. is similar to the benzene oxygenase described earlier. However, detailed report on the enzyme is not available though it is known that it catalyzes the formation of *cis*-1,2-dihydro-1,2-dihydroxy-naphthalene from naphthalene. The *cis*-dihydrodiol was shown to be catalyzed by an NAD^+-dependent dehydrogenase, to 1,2-dihydroxynaphthalene. This enzyme was found to be specific for the *cis*-isomer of the dihydrodiol[49].

The scheme for the oxidation of naphthalene in bacteria and higher organisms is shown in Fig. 3.

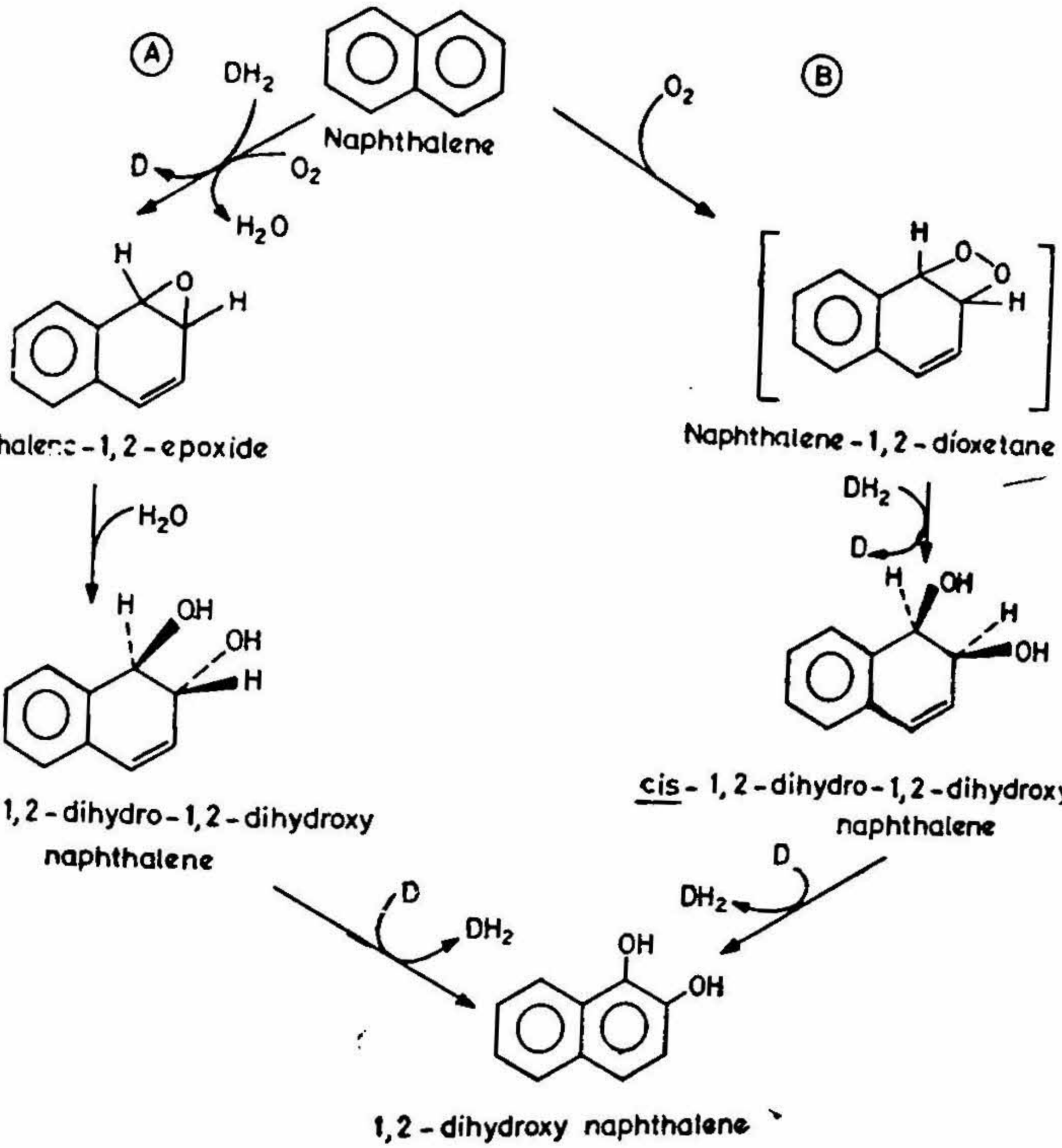


FIG. 3. Metabolism of naphthalene (A) in higher organisms (B) in bacteria.

Double hydroxylation of phenanthrene and anthracene

Earlier studies on the oxidation of phenanthrene by *Pseudomonas*, showed the formation of salicylic acid and 1-hydroxy-2-naphthoic acid as intermediates[58, 59]. Colla *et al.*[60] and Kaneko *et al.*[62] independently identified 3,4-dihydro-3,4-dihydroxy phenanthrene from cultures of *Flavobacterium* metabolizing phenanthrene. Dihydroxyphenanthrene was also proposed by Kiyohara *et al.*[62] as an intermediate in the oxidation of phenanthrene by *Aeromonas*. Evans and his collaborators[63] made an initial observation that phenanthrene is oxidatively metabolized by soil *Pseudomonas* through *trans*-3,4-dihydro-3,4-dihydroxyphenanthrene to 3,4-dihydroxyphenanthrene. Anthracene was similarly shown to be converted to *trans*-1,2-dihydro-1,2-dihydroxyanthracene and then to 1,2-dihydroxyanthracene,

The absolute stereochemistry of the dihydrodiol produced from anthracene by mammals and bacteria was assigned by Akthar *et al.*[64] The *trans*-1,2-dihydro-1,2-dihydroxyanthracene and *cis*-1,2-dihydro-1,2-dihydroxyanthracene were isolated as major metabolites of anthracene from rabbits and *Beijerinckia* respectively. The *cis* and *trans* configuration of the dihydrodiols produced from anthracene and phenanthrene by bacterial and mammalian systems respectively, was also shown by Jerina *et al.*[65] From mutant strains of *Beijerinckia* and *Pseudomonas* which were deficient in dihydrodiol dehydrogenases, they isolated *cis*-3,4-dihydro-3,4-dihydroxyphenanthrene and *cis*-1,2-dihydro-1,2-dihydroxyanthracene. The *cis*-1,2-dihydro-1,2-dihydroxyphenanthrene was also detected as a minor product from phenanthrene. The formation of *trans*-dihydrodiols in mammalian systems was shown by the isolation of epoxide hydrase which converts corresponding arene oxide to *trans*-dihydrodiol[65].

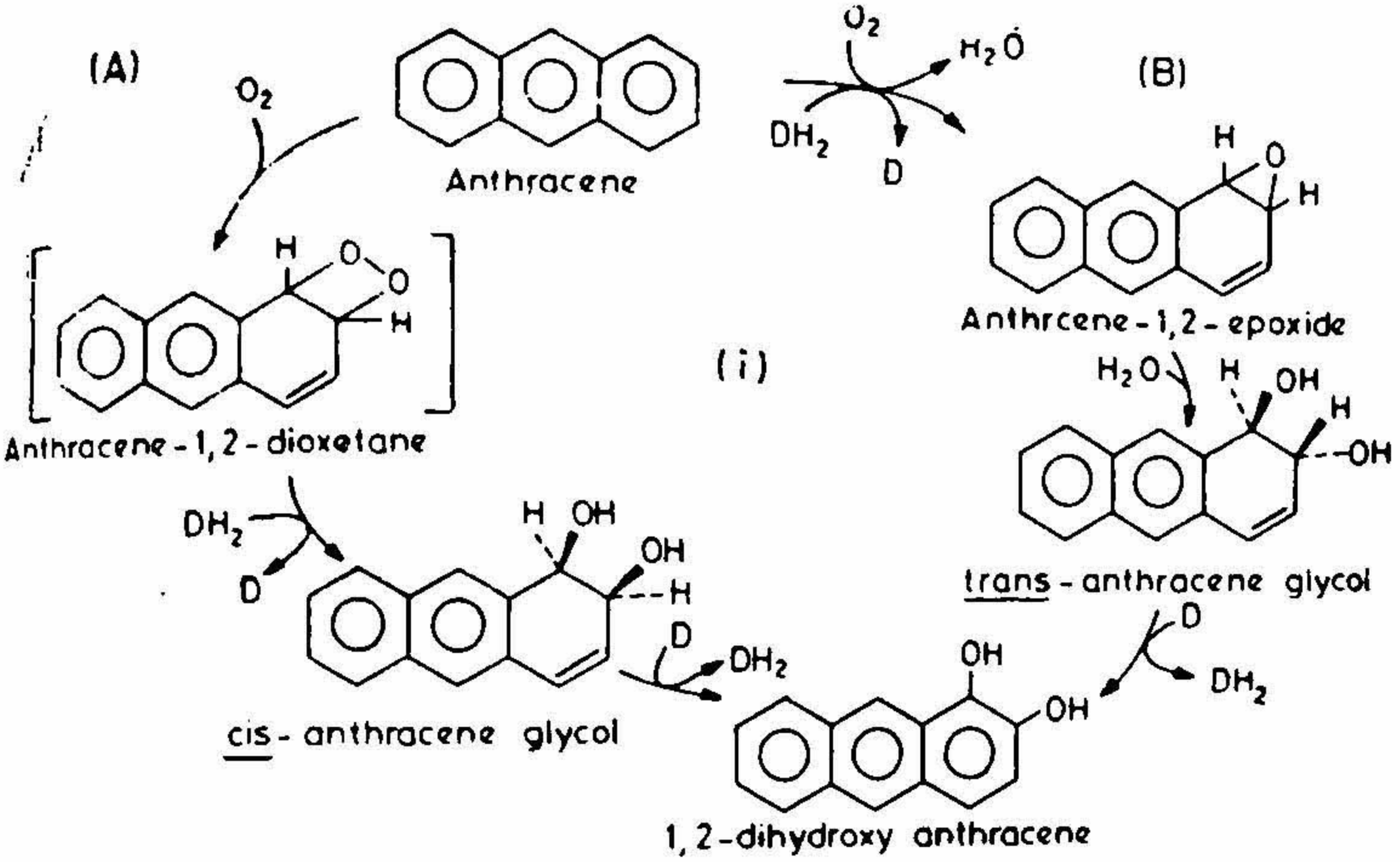
Rodgoff[66] observed that substitution of phenanthrene by a methyl group at '9' position blocks the oxidation of the compound by a resting cell suspension of a phenanthrene-grown soil *Pseudomonas*. When 2-methylphenanthrene was provided, the oxidation rate was observed to be considerably higher. 3-methylphenanthrene is oxidized at an intermediate rate between the above two, even though the methyl group is attached to 'C' directly involved in ring splitting.

These data are in agreement with the hypothesis that polynuclear aromatic hydrocarbons attach to the relevant bacterial enzymes at C–C bonds of high electron density and that ring splitting reactions then occur at other bonds on the substituted molecule. The actual bond that undergoes fission is determined by the electronic and steric configurations of the enzyme-substrate complex. When linearly arranged aromatic compounds such as naphthalene or anthracene are attacked, attachment to the enzyme and ring splitting may take place on the same ring. On the other hand, angular aromatic compounds such as phenanthrene afford attachment to the enzyme at a bond in a region other than the one containing the ring splitting site[66].

Thus the metabolic formation of vicinal dihydrodiols is one of the principal pathways by which both mammals and bacteria oxidize phenanthrene and anthracene. The dihydrodiols of mammalian origin have *trans*-configuration and result from enzymic hydration of arene oxides[61,64,67]. The arene oxides are formed by monooxygenation of the aromatic nucleus. The dihydrodiols of bacterial origin have *cis*-configuration and result from the action of dioxygenases. The dioxygenases that convert anthracene and phenanthrene to their respective dihydrodiols have not been isolated so far. However, the reaction they catalyze is clear from the intermediates isolated. The schematic representation of the degradation of anthracene and phenanthrene by bacterial and mammalian systems is shown in Fig. 4.

Double hydroxylation of toluene and xylene

Two bacterial strains, *Pseudomonas* and *Achromobacter* were shown to grow on toluene as sole carbon source. The toluene-grown cells oxidized 3-methylcatechol without any



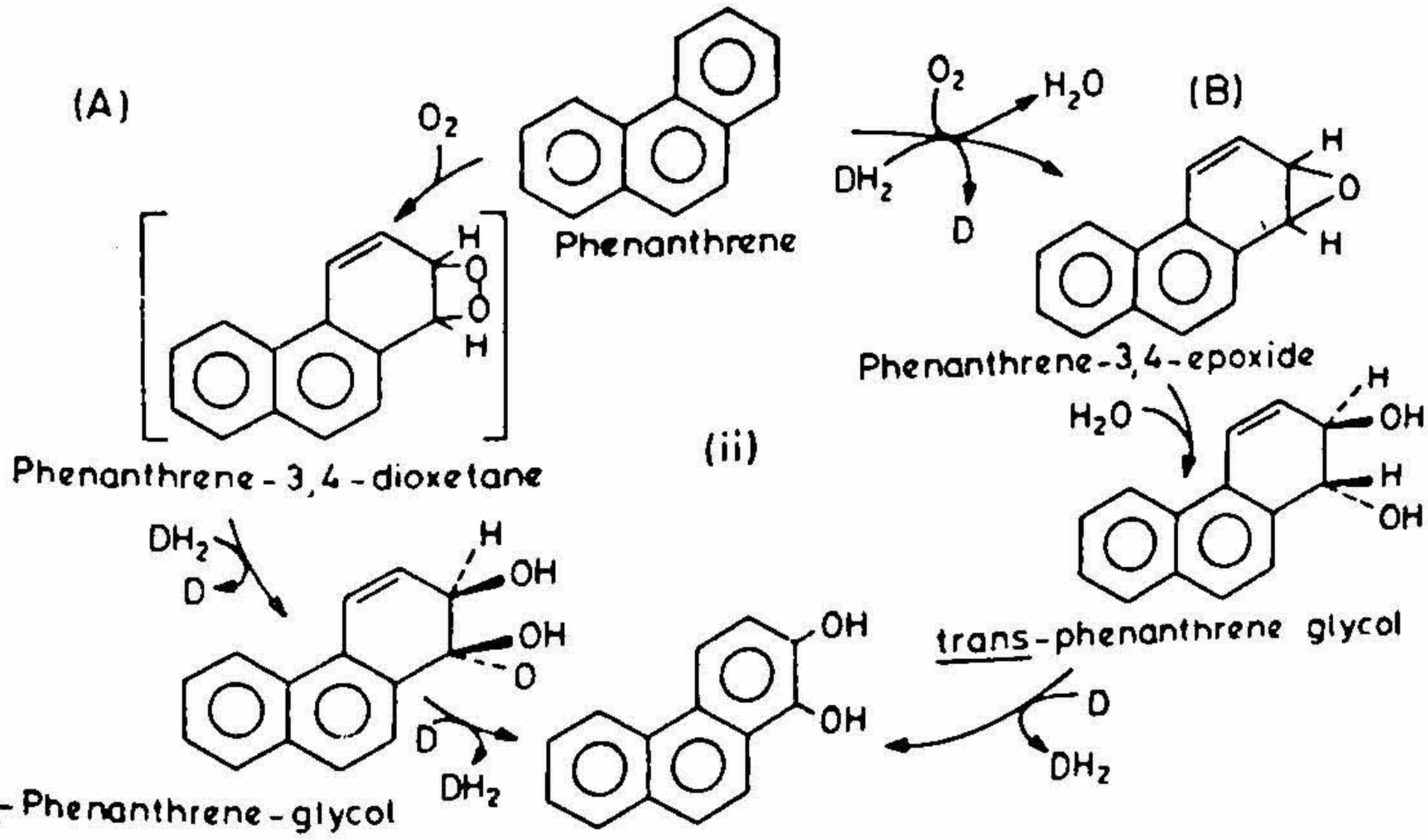


FIG. 4. Oxidation of (i) anthracene and (ii) phenanthrene by (A) bacteria and (B) mammals.

lag period[68]. 3-methylcatechol was also detected in toluene-oxidizing cultures in the early log phase. From the culture medium of a mutant *Pseudomonas*, a neutral compound was isolated by Karplus[69] and identified as 2,3-dihydro-2,3-dihydroxytoluene.

but the stereochemistry of the hydroxyl groups were not established by them. Gibson *et al.*[69] isolated two compounds from a mutant *Pseudomonas* growing on 4-chlorotoluene. These compounds were identified as *cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene and 4-chloro-2,3-dihydroxy-1-methyl benzene. *cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (*cis*-toluene glycol) was also isolated from a mutant *Pseudomonas* growing on toluene[71, 72]. The formation of *cis*-toluene glycol was further confirmed when it was shown to be oxidized to 3-methylcatechol by whole cells[69, 70] and cell-free extracts of *Pseudomonas*[71, 73].

The conversion of toluene to *cis*-toluene glycol is effected by toluene dioxygenase (Fig. 5). Nozaka and Kusunose[74, 75] reported the requirement for three protein fractions for toluene hydroxylation by *Pseudomonas aeruginosa*. A crude extract of the organism was fractionated into three protein components on DEAE-cellulose. With NADH and NAD as cofactors, all the three protein fractions were required for maximum activity. By ammonium sulfate fractionation, two protein components were resolved which had

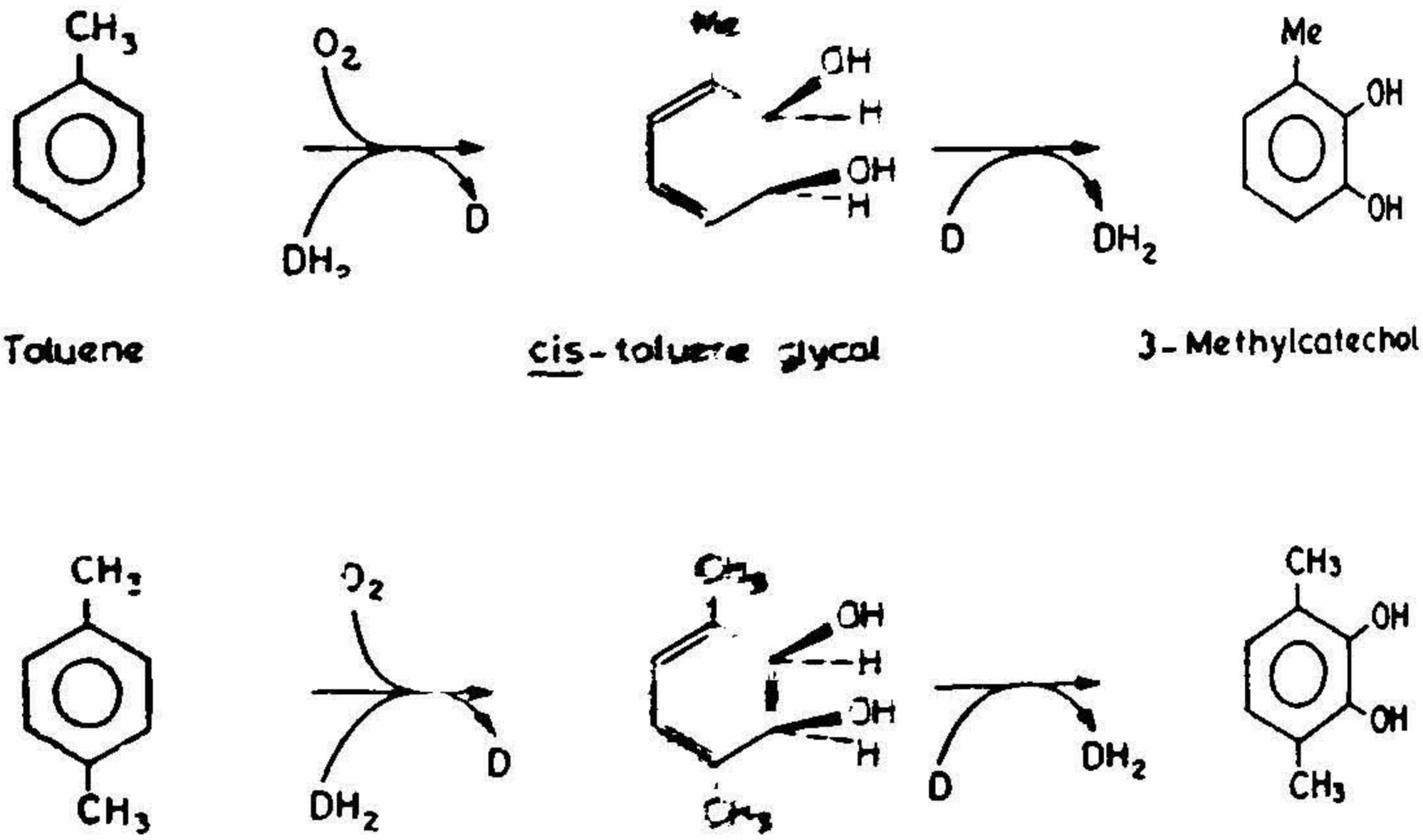


FIG. 5. Scheme for the oxidation of (A) toluene (B) *p*-xylene (C) *m*-xylene.

to be combined for maximum activity. Recently, the multienzyme system, toluene dioxygenase. was purified by Yeh *et al.*[76] and shown to be made up of three protein components. The yellow protein A_1 plays an important role in transfer of electrons to cytochrome *c*. The hydroxylation is stimulated by the presence of the brown protein, B, which has two moles of iron and two moles of inorganic sulfur per mole of protein. The organization of the enzyme is apparently similar to the benzene oxygenase system described earlier. The oxidation of toluene is shown in Fig. 5. The next enzyme in the pathway, *viz.*, *cis*-toluenedihydrodiol dehydrogenase has been purified to homogeneity by Rogers and Gibson[77]. The enzyme is specific for the *cis*-isomer.

The utilization of xylene by *Nocardia corralinas* under co-oxidation conditions was reported by Jamison *et al*[78–80]. *a,a'*-dimethyl-*cis,cis*-muconic acid was found to be the accumulated intermediate. Two pathways were proposed for the degradation of *p*-xylene.

(*a*) A dioxygenation and formation of 2,3-dihydroxy *p*-xylene as intermediate.

(*b*) Degradation of *p*-xylene to *p*-toluic acid and dioxygenation of *p*-toluic acid (This is discussed separately under dihydroxylation of aromatic acids).

Gibson *et al.*[81] isolated an accumulated compound from the culture filtrates of a mutant *Pseudomonas* growing on *p*-xylene as sole carbon source. Similarly another accumulated compound was isolated during the oxidation of *m*-xylene by the organism. These two compounds were identified as *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (*cis-p*-xylenediol) and *cis*-3,5-dimethyl-3,5-cyclohexadiene-1,2-diol. Both the reactions are catalyzed by dioxygenase. It is not clear whether there are separate dioxygenases for the oxidation of *p*- and *m*-xylene or the same dioxygenase catalyzes the oxidation of both substrates. The scheme for the oxidation of *p*- and *m*-xylene is shown in Fig. 5.

Double hydroxylation of biphenyl

The conversion of biphenyl to 2,3-dihydroxybiphenyl in bacterial systems was shown by a number of workers. Lunt and Evans[82] isolated 2,3-dihydroxybiphenyl from pure cultures of gram negative soil bacteria utilizing biphenyl. Catelani *et al.*[83] showed that cultures of *Pseudomonas putida* grown on biphenyl yielded 2,3-dihydro-2,3-dihydroxybiphenyl. By incubation of washed suspensions of *Pseudomonas* with 2,3-dihydroxybiphenyl, further metabolites were isolated. Ring fission was shown to occur between C_1 and C_2 of 2,3-dihydroxybiphenyl[84, 85]. The above compounds were also proposed as intermediates in the degradation of biphenyl and *p*-chlorobiphenyl by *Achromobacter*[86]. However, it was not clear from the above studies whether the dihydro dihydroxy intermediate has a *cis* or *trans* configuration.

Gibson *et al.*[87] isolated a species of *Beijerinckia* that utilizes biphenyl as sole carbon source for growth. *cis*-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene was isolated and

identified from a mutant *Beijerinckia*[88]. Cell-free extracts prepared from the parent organisms oxidized *cis*-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene to 2,3-dihydroxybiphenyl[87]. Hence a *trans*-dihydrodiol intermediate was ruled out.

The scheme for the microbial degradation of biphenyl is shown in Fig. 6. The first step is apparently catalyzed by a dioxygenase which has not been shown in cell-free systems.

Double hydroxylation of benzo (a) pyrene and benzo(a) anthracene

Gibson and his group[89, 90] isolated a mutant strain of *Beijerinckia* which oxidized benzo(a) pyrene and benzo(a) anthracene, when grown on succinate in the presence of biphenyl. The organism was shown to contain an enzyme system that oxidized benzo(a) pyrene and benzo(a) anthracene to a mixture of vicinal dihydrodiols. The major dihydrodiol formed from benzo(a) pyrene was identified as *cis*-9,10-dihydroxy-9,10-dihydrobenzopyrene by comparison with a synthetic sample (Fig. 7). Benzo(a) anthracene was metabolized to four dihydrodiols, the major isomer being *cis*-1,2-dihydro-1,2-dihydroxybenzo (a) anthracene (Fig. 7). On the contrary, higher organisms oxidize the above compounds tc *trans*-vicinal dihydrodiols through arene oxide intermediates[41, 91].

From the available data, it can be generalized that the initial reaction in the bacterial oxidation of aromatic hydrocarbons involves the formation of a *cis*-dihydrodiols, probably *via* a cyclic peroxide type intermediate. In contrast, the eukaryotic organisms oxidize aromatic hydrocarbons to arene oxides which are hydrated to form *trans*-dihydrodiol. It is conceivable that while the prokaryotic organisms employ a dioxygenase to initiate the metabolism of aromatic hydrocarbons, eukaryotic organisms employ a monooxygenase enzyme system. The generalization is summarized in Fig. 8.

3. Double hydroxylation of indole

Sakamoto *et al.*[92] who studied the decomposition of indole in a tapwater bacterium, detected the following compounds in the culture filtrate. Isatin, formylanthranilic acid, anthranilic acid, salicylic acid and catechol. Based on these findings they proposed the following pathway for the degradation of indole. Indole → Indoxyl → dihydroxyindole → Isatin → formylanthranilic acid → anthranilic acid → catechol. Fujioka and Wada[93] isolated a soil microorganism which utilized indole as sole source of carbon and nitrogen. Dihydroxyindole was detected as the intermediate in the metabolism of indole. Direct evidence for the formation of dihydroxyindole as intermediate was not obtained since indole was oxidized to anthranilic acid without the accumulation of dihydroxyindole. However, when skatole was incubated with indole-grown cells, the compound was oxidized with the consumption of one mole of oxygen per mole of the substrate to (+)-2-oxo-3-methyl-3-hydroxyindoline. This compound was not further metabolized in the organism. Skatole does not have a β hydrogen atom and hence enolization of the intermediate formed is not possible. Therefore, ketol accumulates

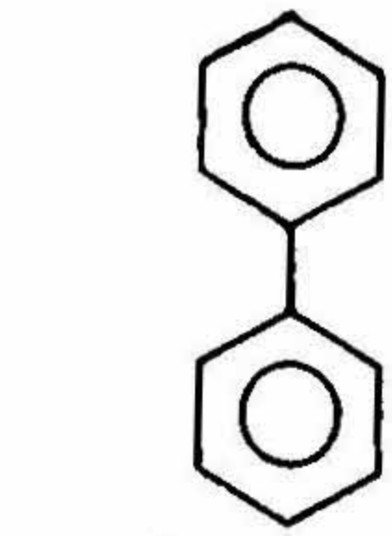


FIG. 6. Bacterial degradation of biphenyl.

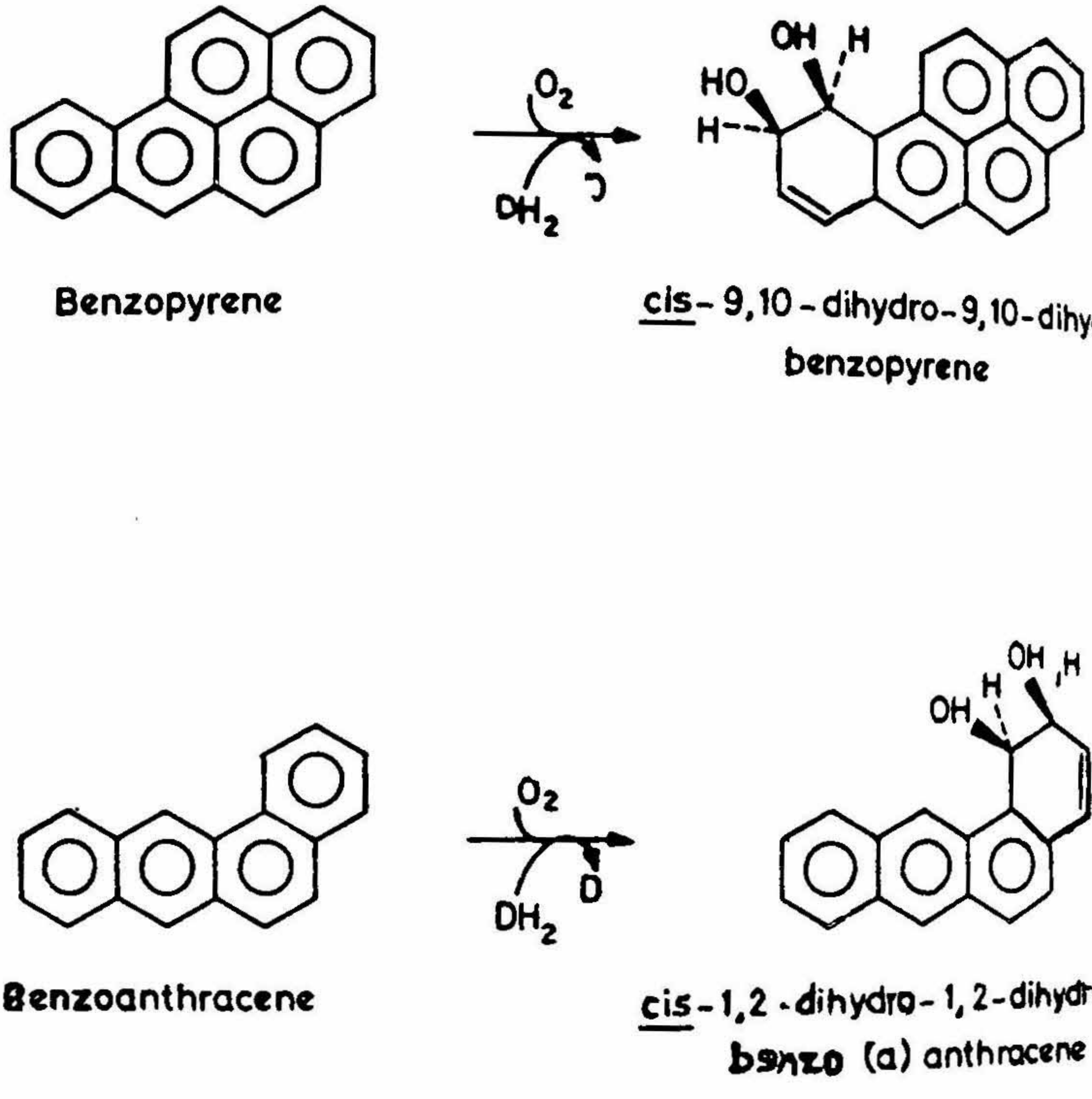


FIG. 7. Oxidation of benzopyrene and benzoanthracene by microorganism.

in the medium. The formation of 2-oxo-3-methyl-3-hydroxyindoline from skatole and the induction of dihydroxyindole oxygenase by indole strongly suggests that indole is metabolized to anthranilic acid *via* dihydroxyindole. Indole oxygenase was also partially purified from the soil microorganism.

It is interesting to note that in the case of indole, no dihydrodiol intermediate is formed as in the case of other aromatic hydrocarbons discussed earlier.

The enzyme catalyzing the oxidation of indole to dihydroxyindole could not be solubilized. The activity was found to be associated with the cellular debris. The oxidation of indole is shown in Fig. 9. Though an epoxide mechanism has been proposed by the authors, a cyclic peroxide intermediate is more likely as the formation of 2-oxo-3-methyl-3-hydroxyindole from skatole could be better explained with the cyclic peroxide intermediate rather than the epoxide intermediate.

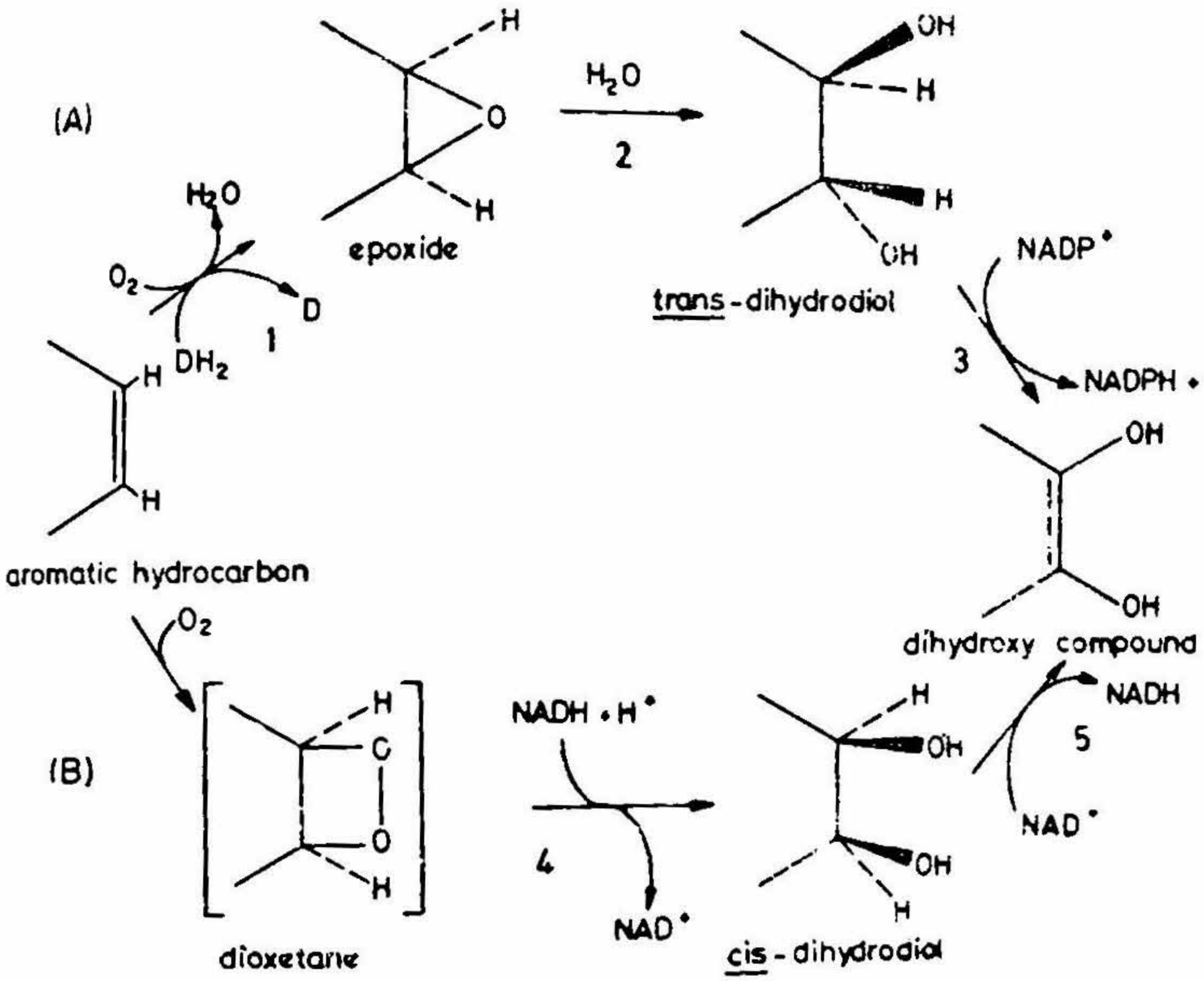


FIG. 8. Initial reactions in the oxidation of aromatic hydrocarbons by (A) higher organisms (B) bacteria.

1. Aromatic hydrocarbon monooxygenase
2. Epoxide hydrase
3. *trans*-dihydrodiol dehydrogenase
4. Aromatic hydrocarbon dioxygenase
5. *cis*-dihydrodiol dehydrogenase

4. Double hydroxylation of pyrazon

Pyrazon, an active ingredient in the herbicide pyramin, was found to be utilized by soil bacteria as sole carbon source. But the metabolites were not identified[94]. DeFrenne *et al.*[95] during their studies on pyrazon degradation by an unidentified bacterium, isolated four metabolites from the culture filtrates. They were identified as:

(*a*) 5-amino-4-chloro-2-(2,3-*cis*-dihydroxycyclohexa-4,6-diene-1-yl)-3(2H)pyridazinone.

(*b*) 2-(5-amino-4-chloro-3-oxo-2,3-dihydro-2-pyridazino)-*cis*, *cis*-muconic acid.

(*c*) 2-pyrone-6-carboxylic acid and

(*d*) 5-amino-4-chloro-3(2H)pyridazinone.

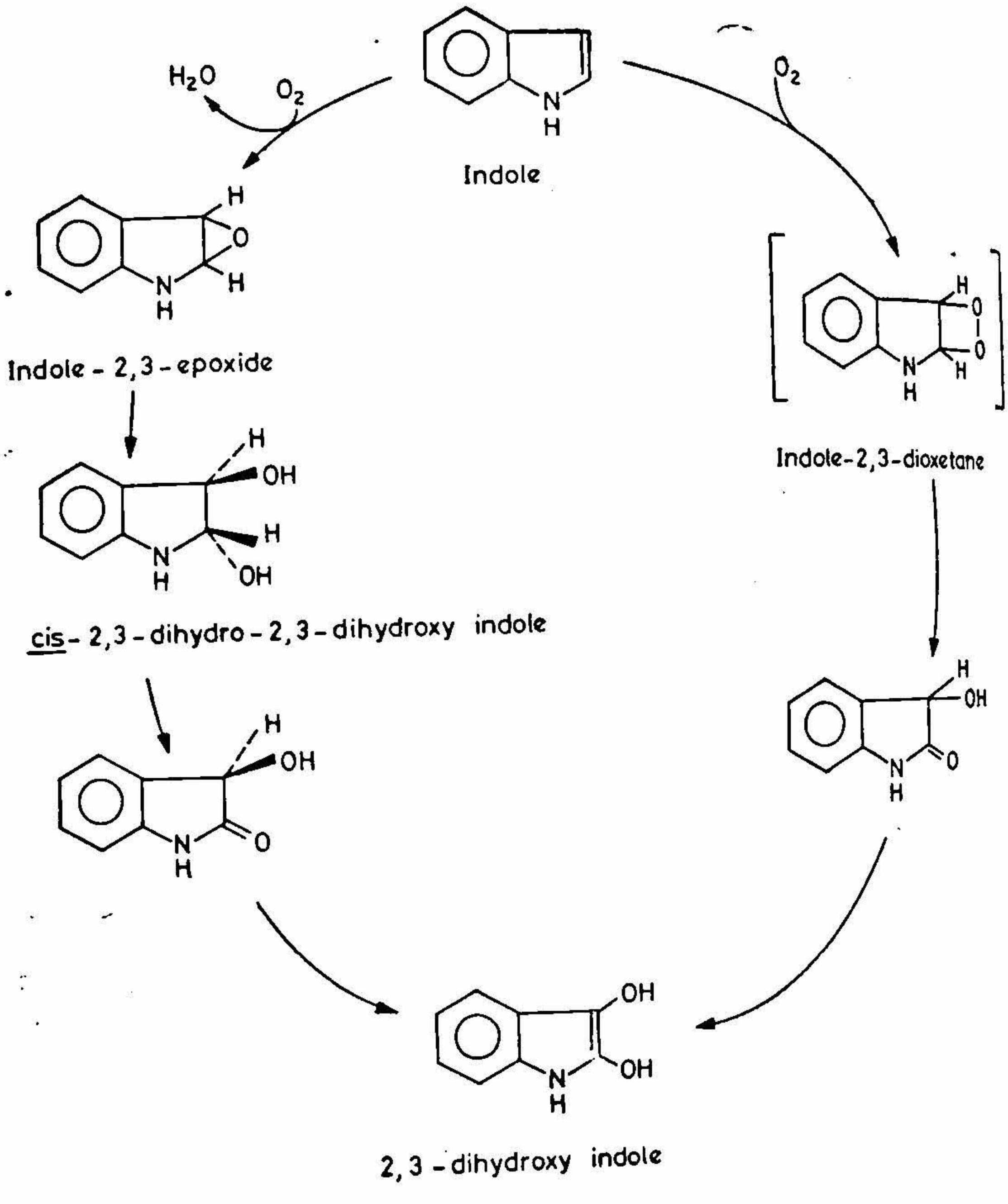


FIG. 9. Oxidation of indole by bacteria.

Compound (*a*) was found to be formed in stoichiometric amounts from pyrazon. The pathway proposed for pyrazon degradation is shown in Fig. 10(*a*). Subsequent studies carried out by Lingen's group[96], led to the isolation of 5-amino-4-chloro-2-(2,3-dihydroxyphen-1-yl)-3(2H)pyridazinone. The compound was also prepared by enzymatic and chemical treatment of 5-amino-4-chloro-2-(2,3-dihydroxycyclohexa-4,6-diene-1-yl) pyridazinone.

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