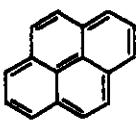
CHAPTER 2
LITERATURE REVIEW

2.1 Pyrene, fluoranthene, phenanthrene and other polycyclic aromatic hydrocarbons (PAHs)

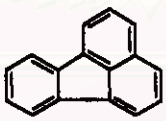
Pyrene, fluoranthene and phenanthrene; the compounds which are included in PAH group as the recalcitrant pollutants and possess hazardous effects to the human being are described their chemical structures and the corresponding properties as well as those of other PAHs.

2.1.1 Chemical structures

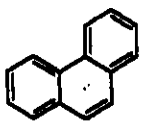
Pyrene and phenanthrene consist of four and three fused benzene rings, respectively, while fluoranthene possesses a structure of three aromatic rings fused with a cyclopentane (Figure 2.1).



Pyrene



Fluoranthene



Phenanthrene

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Figure 2.1 Chemical structures of pyrene, fluoranthene and phenanthrene (Wilson and Jones, 1993)

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In addition to these compounds, other 13 polycyclic aromatic hydrocarbons (PAHs) consist of two or more fused benzene rings in linear, angular or cluster arrangement also constitute a group of priority pollutants (Sutherland *et al.*, 1995). Chemical structures of which are shown in Figure 2.2.

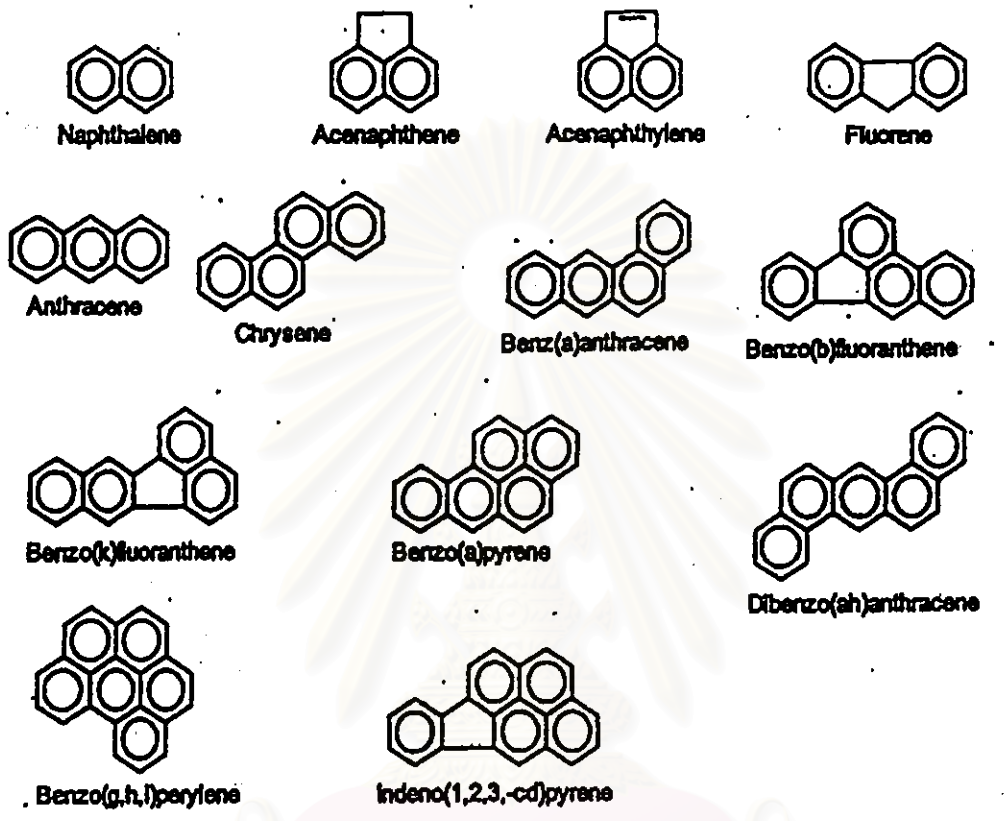

Figure 2.2 Chemical structures of other 13 priority PAHs (Wilson and Jones, 1993)

2.1.2 Source of PAHs

PAHs are ubiquitous in the environment. They occur as by-product of industrial processing, burning of organic materials and during food cooking. Furthermore, they are formed naturally during thermal geologic reactions associated with fossil fuel and mineral production during forest burning. However, anthropogenic sources, especially the burning of fossil fuels are principle source of PAHs that polluted the environment (Wilson and Jones, 1993).

Environment can be contaminated with PAHs by several routes such as leakage of industrial or sewage effluents, accidental discharges during the transport, use and

disposal of petroleum products. Table 2.1 summarizes sources and entrance mechanism of PAHs into the environment (Cerniglia, 1992) which Table 2.2 exemplify level of contamination in certain polluted sites. Data demonstrate that phenanthrene is a predominant PAH found in wood preserving and creosote.

Table 2.1 Major sources and transport mechanism of PAHs in the environment (Cerniglia, 1992; Wilson and Jones, 1993)

Natural oil seep
Refinery and oil storage waste
Accidental spills from oil tankers and other ships
Municipal and urban wastewater and discharge runoff
River-borne pollution
Atmospheric fallout of fly ash particulates
Petrochemical industrial effluents
Coal tar and other coal processing wastes
Automobile engine exhausts
Combustion of fossil fuels (gasoline, kerosene, coal, diesel fuel)
Smoked, charcoal broiled, or pan fried foods
Tobacco and cigarette smoke
Forest and prairie fires
Rural and urban sewage sludge
Refuse and waste incineration
Coal gasification and liquefaction processes
Creosote and other wood preservative wastes
Commercial and pleasure boating activities
Coke production
Catalytic cracking
Carbon-black production and use
Asphalt production and use
Landfill and waste dumps

Table 2.2 Concentrations of PAHs at contaminated sites (Wilson and Jones, 1993)

PAH	Wood-preserving[a]		Creosote Production[b]		Wood treatment[c]	Coking Plant[c]	Coking plant[d]	Gas works[a]	
	Surface-soil	Subsoil	Mean	range				mean	range
Naphthalene	1	3925	1313	<1-5769	91.8	56	59		
1-Methyl naphthalene	1	1452	901	<1-1617			87		
2-Methyl naphthalene	1	623	482	6-2926			112		
2,6 - Dimethyl naphthalene	2	296							
2,3-Dimethyl naphthalene	1	168							
Acenaphthylene	5	49	33	6-77			187		
Accenaphthene	7	1368					29	2	0-11
Fluorene	3	1792	650	49-1294	620	7	245	225	113-233
Phenanthrene	11	4434	1595	76-3402	1440	27	277	379	150-716
Anthrecene	10	3037	334	15-693	766	6	130	156	57-295
2-Methyl anthracene	14	516							
Fluoranthene	35	1629	682	21-1464	1350	34		2174	614-366
Pyrene	49	1303	642	19-1303	983	28	285	491	170-833
2,3-Benzo (b) fluorene	8	288							
Chrysene	38	481	614	8-1586	321	11	135	345	183-59
Benzo (a) pyrene	28	82			93.7	14		92	45-159
Benzo (a) anthracene	12	171			356	16	200	317	155-39
Benzo (b) fluoranthene and	38	140						260	108-55
Benzo (k) fluoranthene								238	152-44
Dibenz (ah) anthracene					10-1	2		2451	950-38
Indeno (123 cd) pyrene	10	23						207	121-31

All concentrations in mg/kg dry matter

[a] Mueller *et al.*,1991a,b-composite sample

[b] Ellis *et al.*,1991-samples are 1.5 m or 3.5 m

[c] Weissenfels *et al.*,1990b

[d] Werner and Brauch, 1988

[a] Bewley *et al.*,1989-samples taken from prototype treatment bed

2.1.3 Toxicological characteristics

The increase of PAHs in environment is of great concern due to the fact that some low-molecular-weight PAHs are acutely toxic (Darvill and Wilhm, 1984), in addition most higher-molecular-weight compounds could also exert their mutagenic, teratogenic and potential carcinogenic effects (International Agency for Research on Cancer [IARC] 1983). As a result of these, the U.S. Environmental Protection Agency (EPA) has listed many of PAHs as the priority pollutants (Patnaik, 1992).

Toxicological characteristics of 16 PAHs classified as priority pollutants are summarized (Table 2.3).

Table 2.3 Physical properties and Toxicological characteristics of 16 PAHs priority pollutants (Patnaik, 1992)

PAHs	Formula; MW	Physical properties	Health hazard
Pyrene	$C_{16}H_{10}$; 202.26	Colorless monoclinic prisms crystallized from alcohol, yellowish due to the presence of tetracene; produces slight blue fluorescence ; melting point 156 °C; boiling point 404°C; insoluble in water, soluble in organic solvents.	Inhalation of its vapors or ingestion caused irritation of the eyes, excitement and muscle contraction in rats and mice. An oral LD_{50} in mice is 800 mg/kg. Studies on experimental animals do not give evidence of carcinogenicity. Skin tumors, however, have been reported in mice.
Fluoranthene	$C_{16}H_{10}$; 202.26	Plates crystallized from alcohol; melting point 110 °C ; sublimes; insoluble in water, moderately soluble in alcohol, dissolves in most other organic solvents.	Fluoranthene exhibited mild oral and dermal toxicity in animals. The acute toxicity is lower than that of phenanthrene. An oral LD_{50} in rats is 2000 mg/kg. It may cause tumor in skin at the site of application. However, any carcinogenic action from this compound in animals is not known.

Table 2.3 (continued)

PAHs	Formulary; MW	Physical properties	Health hazard
Phenanthrene	$C_{14}H_{10}$; 178.24	Monoclinic plates crystallized from alcohol; isomeric with anthracene; shows blue fluorescence in solution; melting point 100 °C; boiling point 340 °C; sublimes in vacuum; insoluble in water, moderately soluble in alcohol, dissolves readily in benzene, toluene, chloroform, carbon disulfide and anhydrous ether.	The acute oral toxicity of phenanthrene is low. It is more toxic than anthracene. An oral LD_{50} in mice is 700 mg/kg. It may cause tumor in skin at the site of application.
Naphthalene	$C_{10}H_8$; 128.18	White volatile crystalline flakes with a strong aromatic odor; melting point 80.2°C; boiling point 218 °C; insoluble in water, dissolves in most organic solvents.	Inhalation of naphthalene vapor may cause irritation of the eyes, skin and respiratory tract, and injury to the cornea. Other symptoms are headache, nausea, confusion, and excitement. The most severe toxic effects from naphthalene, however, may come from oral intake of large doses of this compound. In animals and humans, ingestion of large amounts may cause acute hemolytic anemia and hemoglobinuria. Other symptoms are gastrointestinal pain and kidney damage.
Acenaphthene	$C_{12}H_{10}$; 154.22		Carcinogenicity of acenaphthene in animals is not established. Tests for mutagenicity have given inconclusive results.

Table 2.3 (continued)

PAHs	Formula; MW	Physical properties	Health hazard
Acenaphthylene	$C_{12}H_8$; 152.20		Carcinogenic properties of acenaphthylene in animals or humans a not known. Its toxicity data are not available.
Fluorene	$C_{13}H_{10}$; 166.23	White leaflets from alcohol; melting point 116°C; sublimes in vacuum; insoluble in water, moderately soluble in hot alcohol, dissolves readily in most other organic solvents.	Acute toxicity in animals is very low. An intraperitoneal LD_{50} in mice is 2000 mg/kg. Carcinogenicity of fluorene in animals is not well established.
Anthracene	$C_{14}H_{10}$; 178.24		Carcinogenicity of anthracene is not known. Its toxicity is very low. An intraperitoneal LD_{50} in mice is 430 mg/kg.
Chrysene	$C_{18}H_{12}$; 228.30	Orthorhombic bipyramidal plates crystallized from benzene; melting point 254 °C; boiling point 488°C; insoluble in water, slightly soluble in alcohol, slightly soluble in cold organic solvents, moderately soluble in these solvents when hot.	There is very little information published on the acute toxicity of chrysene. Animal studies show sufficient evidence of carcinogenicity. It produced skin cancer in animals. Subcutaneous administration of chrysene in mice caused tumors at the site of application.
Benzo(a) anthracene	$C_{18}H_{12}$; 228.30	Crystallizes as plates from glacial acetic acid or alcohol; produces greenish-yellow fluorescence; melting point 254 °C; boiling point 488°C; sublimes; insoluble in water, slightly soluble in alcohol, dissolves in most other organic solvents.	Its carcinogenic actions in animals is well established. Subcutameous administration of this compound in mice resulted in tumors at the sites of application.

Table 2.3 (continued)

PAHs	Formula; MW	Health hazard
Benzo(b) fluoranthene	$C_{20}H_{12}$; 252.32	There is sufficient evidence on the carcinogenicity of this compound in animals. It produced tumors at the site of application. Cancers in lungs and skin have been observed in animals.
Benzo(k) fluoranthene	$C_{20}H_{12}$; 252.32	This compound caused lungs and skin cancers in animals. It produced tumors at the site of application. Its carcinogenicity in humans is not known.
Benzo(a) pyrene	$C_{20}H_{12}$; 252.32	Animal studies show sufficient evidence of its carcinogenicity by all routes of exposure affecting a variety of tissues, including the lungs, skin, liver, kidney and blood. Besides, it exhibited teratogenic effects in test species. It is a mutagen. It showed positive in a histidine reversion-Ames test, cell transform mouse embryo test, and in in vitro SCE-human lymphocytes.
Dibenzo(a,h) anthracene	$C_{22}H_{14}$; 278.36	The toxicity of this compound is on the same order as that of Benzo(a) pyrene.
Benzo(g,h,i) perylene	$C_{22}H_{12}$; 276.34	This compound is expected to show carcinogenic properties, based on its structural similarities with other carcinogenic PAHs.
Indeno(1,2,3,-cd)pyrene	$C_{22}H_{12}$; 276.34	This compound causes lung cancer in animals. There is sufficient evidence of its carcinogenic actions in animals.

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2.2 Biodegradation of PAHs by microorganisms

In the past decade attempts has been made for the use of bioremediation, a process using microorganisms to break down hazardous organic materials to harmless compound (Baker and Herson, 1994) as means for treating hydrocarbon-contaminated site e.g. soil (Blackburn and Hafker, 1993).

Many reports cited microbiological degradation of PAHs major process that could effectively decontaminate the environment while these compounds could be mineralized or partially transformed by microorganism (Cerniglia, 1992).

2.2.1 Microbial mineralization

Complete mineralization is the process enable bacteria to grow on PAHs as sole source of carbon and energy. In doing so the organism converts the respective substrates to CO_2, cell components and typical products of the usual catabolic pathway (Alexander, 1994). This degradation plays an important role to decontaminate the environment without leaving behind the toxic compounds or intermediates formed from the PAHs degradation. The isolation of bacteria capable of utilizing individual PAHs has been well documented such as Pseusomonads and *Aeromonas* sp. which were found to be able to mineralize phenanthrene (Evans *et al.*, 1965; Kiyohara *et al.*, 1976), *Alcaligenes denitrificans* could grow on fluoranthene as sole carbon and energy (Weissenfels *et al.*, 1990a and 1991), while *Rhodococcus* sp. and *Mycobacterium* sp. could mineralize pyrene (Waler *et al.*, 1991 and Boldrin *et al.*, 1993).

2.2.2 Microbial co-metabolism

PAHs can be co-metabolized by microorganisms which transform them without utilizing as sole carbon and energy sources (Alexander, 1980 and Horvath, 1972). In these organisms, enzymes involved in PAH degradation are not or only limitedly induced by the PAH-compound but it acts as a cosubstrate. Those cosubstrates are transformed by enzymes induced by growth substrate which is structurally related with

the cosubstrate PAH compounds. Co-metabolism seems to be related to the specificity of the enzymes since the microorganisms not necessarily have sufficient array of enzymes to bring about the complete mineralization of the cosubstrate. Furthermore, metabolites from PAH degradation may accumulate or used as cosubstrate by other microorganism (Alexander, 1980). It was proposed that co-metabolism might be an important mechanism to degrade mixtures of PAHs and higher molecular weight PAHs (Mueller *et al.*, 1989 and Pritchard,1995).

Several researchers have reported the ability of bacteria to co-metabolize the recalcitrant compounds. *Pseudomonas putida* grown on naphthalene was able to co-metabolize fluoranthene (Bamsley, 1975) and *Alcaligenes denitrificans* WW1 could co-metabolize pyrene in the presence of fluoranthene (Weissenfels *et al.*, 1991). *Mycobacterium* sp. strain PYR-1 and RJGII-135 co-metabolized pyrene and fluoranthene or only pyrene when grew in mineral salts medium supplemented with peptone, yeast extract and soluble starch (Heitkamp *et al.*, 1988 and Grosser *et al.*, 1991). Bouchez *et al.* (1995) demonstrated that unidentified bacterium *S Phe Na 1* could co-metabolize fluoranthene in the presence of phenanthrene. Beckles *et al.* (1997) revealed that fluoranthene was degraded when mixed with naphthalene. Phenanthrene stimulated microbial growth and the degradation of dibenz(a,h) anthracene as well as benzo(a)pyrene when added to cultures containing these compounds (Juhasz *et al.*, 1997).

2.3 Mechanisms for biodegradation of PAHs by bacteria

2.3.1 General pathways of PAHs metabolism

The biodegradation of PAHs could take place under both aerobic and anaerobic conditions. However, since the anaerobic biodegradation of PAHs is a slow process (Harayama, 1997) more attention is focusing on the aerobic biodegradation.

In bacteria, biodegradation of PAHs always initiate by the introduction of both oxygen atoms into the aromatic nucleus, forming a *cis*-dihydrodiol which catalyzed by a multicomponent dioxygenase. The resulting compounds are rearomatised through a

cis-dihydrodiol dehydrogenase to form dihydroxylated derivatives. These catechol-like substrates can be further cleaved by dioxygenase via the *ortho* fission (intradiol pathway), which take place between the two carbon atoms possessing hydroxyl groups to form *cis,cis*-muconic acid. On the other hand, by means of *meta* fission (extradiol pathway) cleavage of bond between a carbon atom with a hydroxy group and the adjacent carbon atom could take place (Figure 2.3) (Cerniglia, 1992).

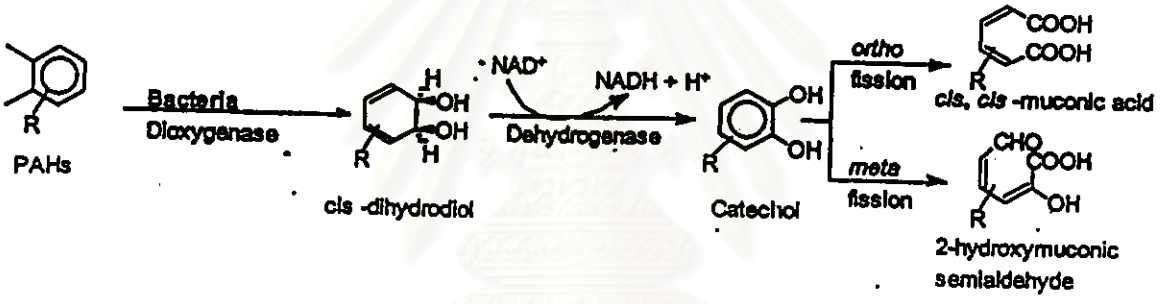

Figure 2.3 General pathways for bacterial catabolism of PAHs (Cerniglia, 1992)

2.3.2 Naphthalene degradation pathways

The metabolism of naphthalene, low molecular weight PAH, by bacteria has been extensively studied more than that of other PAHs. Thus naphthalene metabolism is used as a model for studies on the metabolism carcinogenic PAHs. The first report about the naphthalene oxidation mechanism in Pseudomonads was presented by Davies and Evans (1964). Afterward numerous works have been documented for instance, Ryu *et al.* in 1989 reported on naphthalene metabolism in *Acinetobacter calcoaceticus*. *Mycobacterium* sp. and *Rhodococcus* sp. were also found to be capable of mineralizing naphthalene (Heitkamp *et al.*, 1989, Kelley *et al.*, 1990 and Walter *et al.*, 1991). The pathway for naphthalene degradation was eventually proposed as follow (Figure 2.4).

Naphthalene via the catalysis of naphthalene dioxygenase is initially oxido-reduced to form *cis*-1,2-dihydroxy-1,2-dihydroxynaphthalene (Ensley *et al.*, 1983). Further reaction is the transformation of *cis*-1,2-dihydroxy-1,2-dihydroxynaphthalene by *cis*-naphthalene dihydrodiol dehydrogenase to 1,2-dihydroxynaphthalene, which is subsequently oxidized by 1,2-dihydroxynaphthalene oxygenase to form 2-hydroxychromene-2-carboxylic acid (HCCA). The next conversion is catalyzed by isomerase to form *cis*- and *trans*-o-hydroxybenzylidenepyruvic acid (tHBPA). This compound is then cleaved by tHBPA hydratase and aldolase into salicylaldehyde and pyruvate. Salicylaldehyde is further oxidized to salicylic acid by a dehydrogenase (Davies and Evans, 1964; Eaton and Chapman,1992).

Salicylic acid is further mineralized via three major pathways namely are *meta* pathway, *ortho* or β-ketoadipate pathway (Yen and Serdar, 1988) and gentisate pathway (Sutherland *et al.*, 1995).

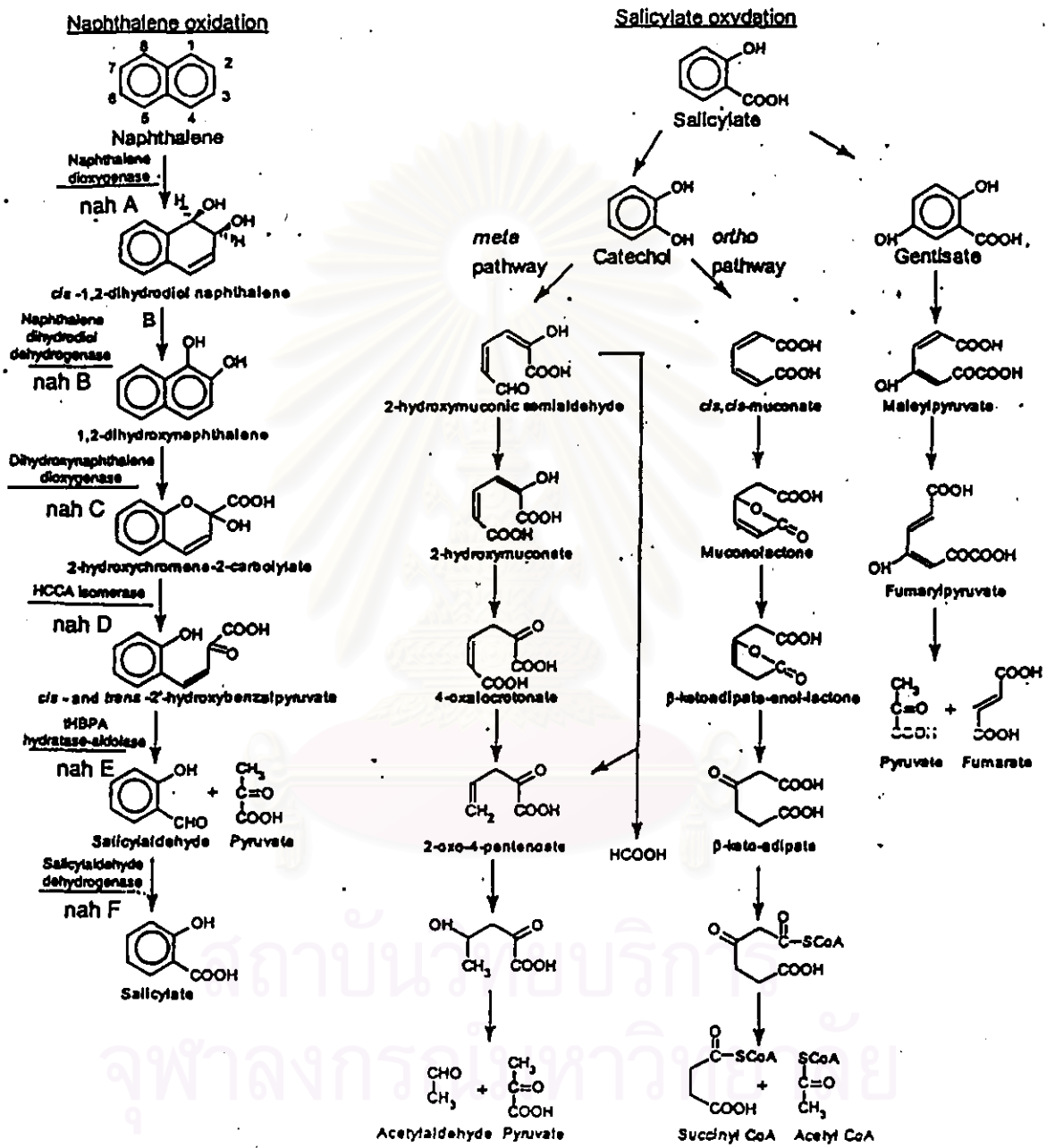

Figure 2.4 Naphthalene and salicylate pathways in Pseudomonads (Evans *et al.*, 1964; Eaton and Chapman; 1992; Yen and Serdar, 1988; Sutherland *et al.*, 1995)

2.3.3 Phenanthrene degradation pathways

The catabolism of phenanthrene by bacteria has been studied in many microorganism. Generally, phenanthrene can be metabolized by initial dioxygenation at 3,4-positions (Evans *et al.*, 1965; Kiyohara,1976 and 1978). Most metabolites in this pathway have already been characterized. Pseudomonads metabolized phenanthrene through phenanthrene *cis*-3,4-dihydrodiol which is dehydrogenated to form 3,4-dihydroxynaphthalene, followed by ring-cleavage by dioxygenase and isomerase to *cis*-4-(1-hydroxy-naphth-2-yl)-2-oxobut-3-enoic acid and converted into 7,8-benzocoumarin or to 1-hydroxy-2-naphthoic acid by hydratase, aldolase and dehydrogenase. The products obtained are further oxidatively decarboxylated to 1,2-dihydroxynaphthalene (Evans *et al.*, 1965). Thus this compound is mineralized through salicylate and catechol via naphthalene degradation pathway (Davies and Evans, 1964). On the other hand Kiyohara *et al.* (1976) proposed the alternative phenanthrene degradation pathway in *Aeromonas* sp. in which 1-hydroxy-2-naphthoic acid can be oxidized through 2-carboxybenzaldehyde, o-phthalate and protocatechuate. This pathway was found in many other bacteria including, Pseudomonads, Vibrios (Kiyohara *et al.*, 1978) *Mycobacterium* sp. BG1 (Guerin and Jones, 1988) and *Alcaligenes faecalis* AFK2 (Kiyohara *et al.*, 1982). Pathway for phenanthrene metabolism in bacteria is shown in Figure 2.5.

Despite above finding, little is known about the degradation via initial dioxygenation at the 1 and 2 position of phenanthrene. Previously, Jerina *et al.* (1976) could detect *cis*-1,2-dihidroxy-1,2-dihydrophenanthrene as a minor product of phenanthrene metabolism in the mutant strains, *Beijerinckia* B-836 and *Pseudomonas putida* 119 which are dihydrodiol dehydrogenase deficiencies. Moreover, Kiyohara *et al.* (1994) demonstrated that *P. putida* AC10 carrying pIP7 containing *pah* genes of *P. putida* OUS82 could convert phenanthrene with major product of 1-hydroxy-2-naphthohic acid and small amount of 2-hydroxy-1-naphthohic acid. These finding suggested the possibility that certain bacteria could degrade phenanthrene via initial dioxygenation at the 1 and 2 positions though it may serve as minor pathway.

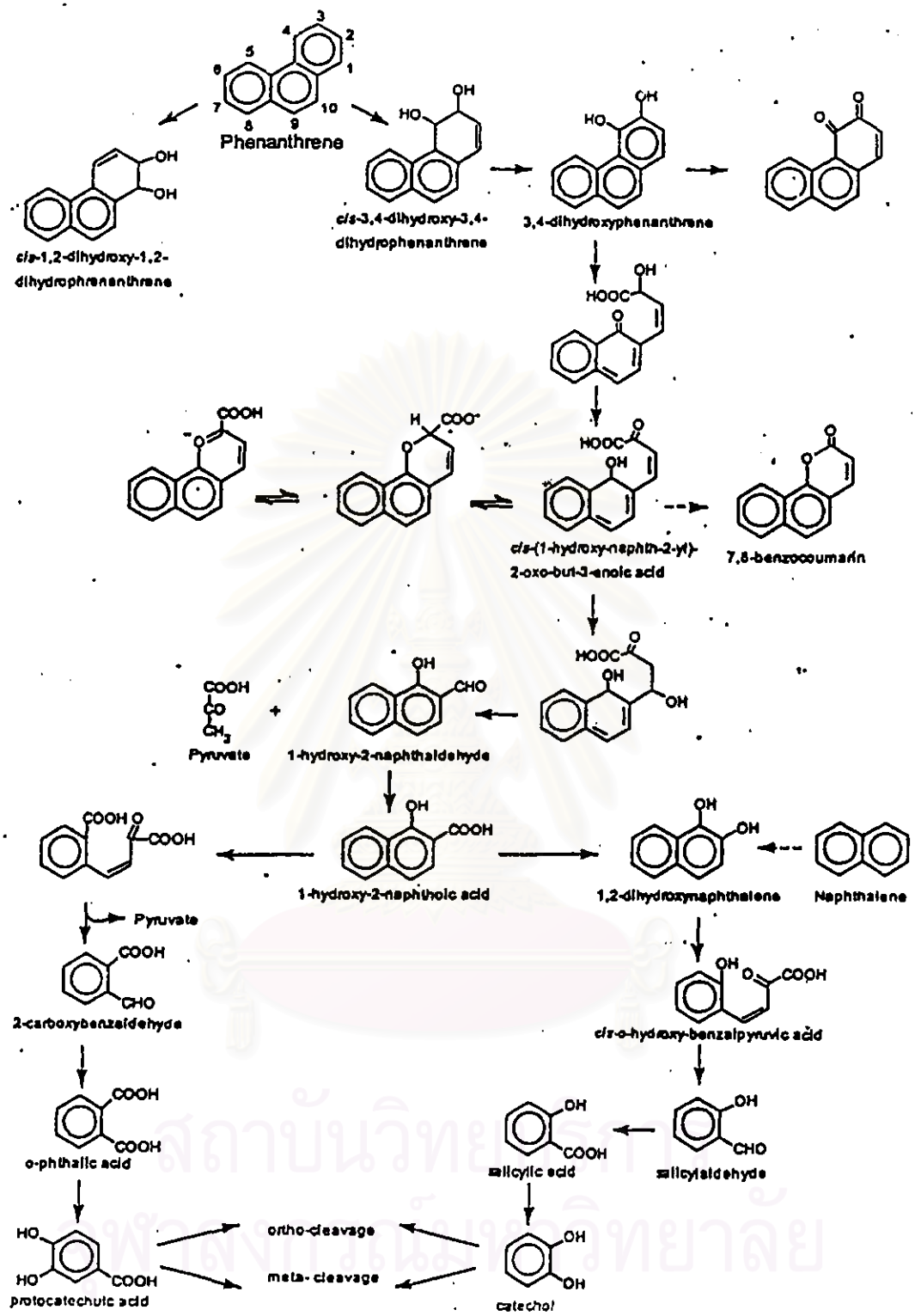

Figure 2.5 Pathway of phenanthrene metabolism by bacteria (Evans *et al.*, 1965; Kiyohara *et al.*, 1976; Jerina *et al.*, 1976)

2.3.4 Pyrene degradation pathways

Pyrene could be catabolized by *Mycobacterium* spp. (Boldrin *et al.*, 1993, Dean-Ross and Cerniglia 1996; Rehmann *et al.*, 1999), *Gordona* sp. (Kästner *et al.*, 1994), *Rhodococcus* sp (Walter *et al.*, 1991 and Bouchez *et al.*, 1997) and *Pseudomonas* sp. (Thibault *et al.*, 1996). These bacteria utilized pyrene as source of sole carbon and energy. Pyrene catabolic pathways have been proposed in two of these strains (Figure 2.6). In *Rhodococcus* sp. UW1 initial oxidation of pyrene took place at the 1,2- or 4,5-positions. Many intermediates from both routes such as 1-hydroxypyrene and 4-hydroxyperinaphthenone have been identified. In *Mycobacterium flavescens* the metabolites from oxidation took place at 4,5-positions, the resulting products; *cis*-4,5-pyrene dihydrodiol, 4,5-phenanthrenedioic acid, 4-phenanthroic acid and phthalic acid, have been isolated. Furthermore, pyrene degradation pathways in bacteria which co-oxidize this compound have also been established. The co-oxidation of pyrene to yield *cis*-and *trans*-dihydrodiols and pyrenols by *Mycobacterium* sp. PYR-1 is reported to be catalyzed by dioxygenase as well as monooxygenase (Heitkamp *et al.*, 1988b). Other major metabolites identified were 4-hydroxyperinaphthenone, 4-phenanthroic acid, cinnamic acid and phthalic acid. *Mycobacterium* sp. RJGII-135 (Grosser *et al.*, 1991) could co-oxidize pyrene to from 4,5-pyrene dihydrodiol, 4,5-phenanthrenedioic acid and 4-phenanthroic acid (Schneider *et al.*, 1996).

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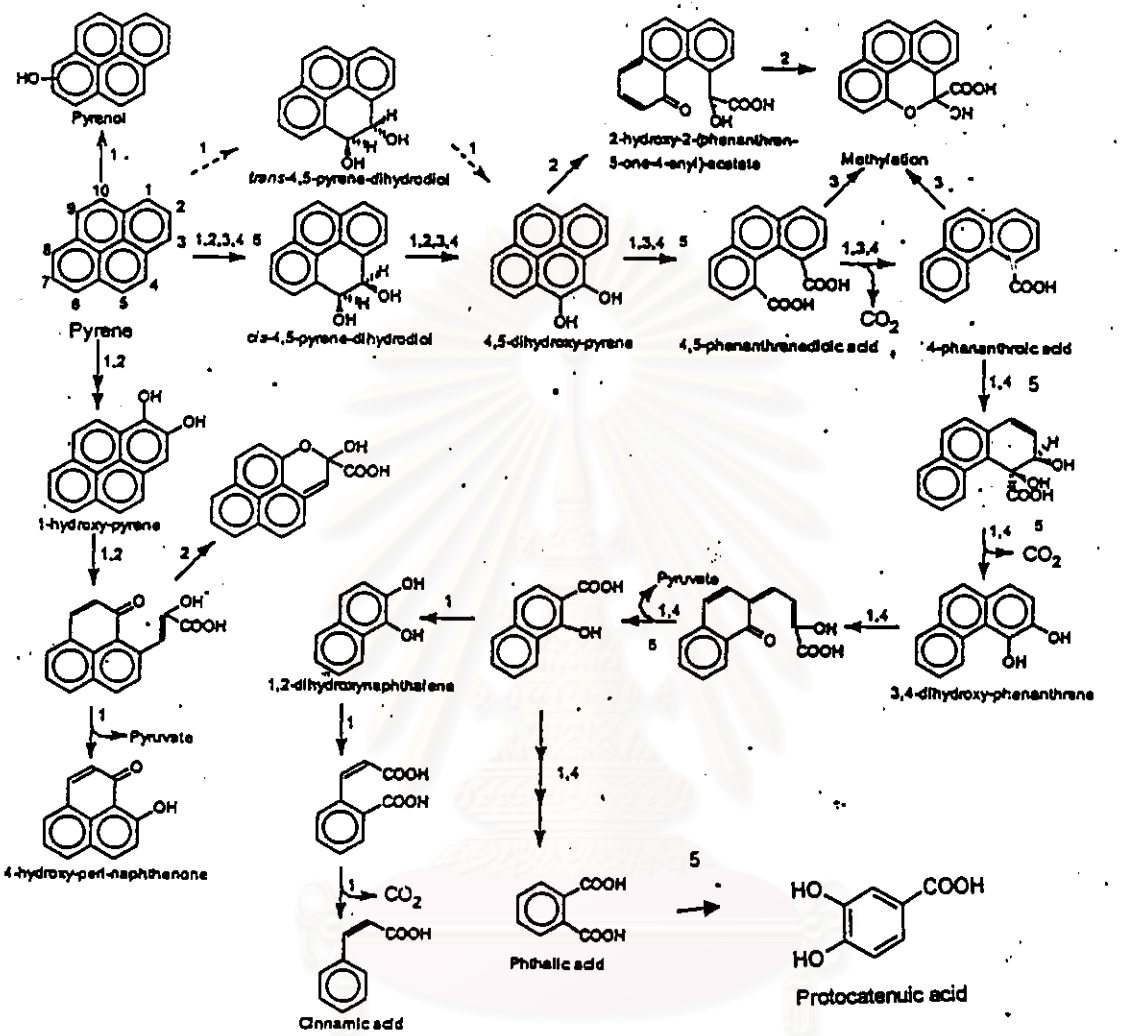

1. *Mycobacterium* sp. PYR-1 (Heitkamp *et al.*, 1988b; Cerniglia, 1992)
2. *Rhodococcus* sp. UW1 (Walter *et al.*, 1991)
3. *Mycobacterium* sp. RJGII-135 (Grosser *et al.*, 1991; Schneider *et al.*, 1996)
4. *Mycobacterium flavescens* ATCC700033 (Dean-Ross and Cerniglia, 1996)
5. *Mycobacterium* sp KP7 (Rehmann *et al.*, 1999)

Figure 2.6 Pathway of pyrene metabolism by bacteria

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2.3.5 Fluoranthene degradation pathways

For the degradation of fluoranthene, the compound could initially be attacked by bacteria at the 1,2-, 7,8- or 9,10- positions. In *Alcaligenes denitridicans*, fluoranthene is used as sole carbon and energy source and the initial attack takes place at 9,10-positions to yield 7-acenapthenone, 7-hydroxyacenaphthylene and 3-hydroxymethyl-4,5-benzocoumarin (Weissenfels *et al.*, 1990a and 1991). Besides, *Mycobacterium* sp. PYR-1 uses different three pathways to co-oxidize fluoranthene (Heitkamp *et al.*,1988b). Many metabolites for example; 9-fluorenone-1-carboxylic acid, 8-hydroxy-7-methoxyfluoranthene, 9-fluorenol, 9-fluorenone, 1-acenaphthone, 9-hydroxy-1-fluorenecarboxylic acid, phthalic acid, 2-carboxybenzaldehyde, benzoic acid, phenylacetic acid and adipic acid were identified (Kelley *et al.*, 1991 and 1993). Fluoranthene degradation pathway is shown in Figure 2.7. Other bacteria such as *Rhodococcus* sp. UW1 (Walter *et al.*, 1991), *Mycobacterium* sp. BB1 and VF1 (Boldrin *et al.*, 1993 and Kästner *et al.*, 1994) can also utilize fluoranthene as a sole source of carbon and energy. Furthermore, *Pseudomonas* spp. growing on naphthalene is able to co-metabolize fluoranthene (Barnsley, 1975).

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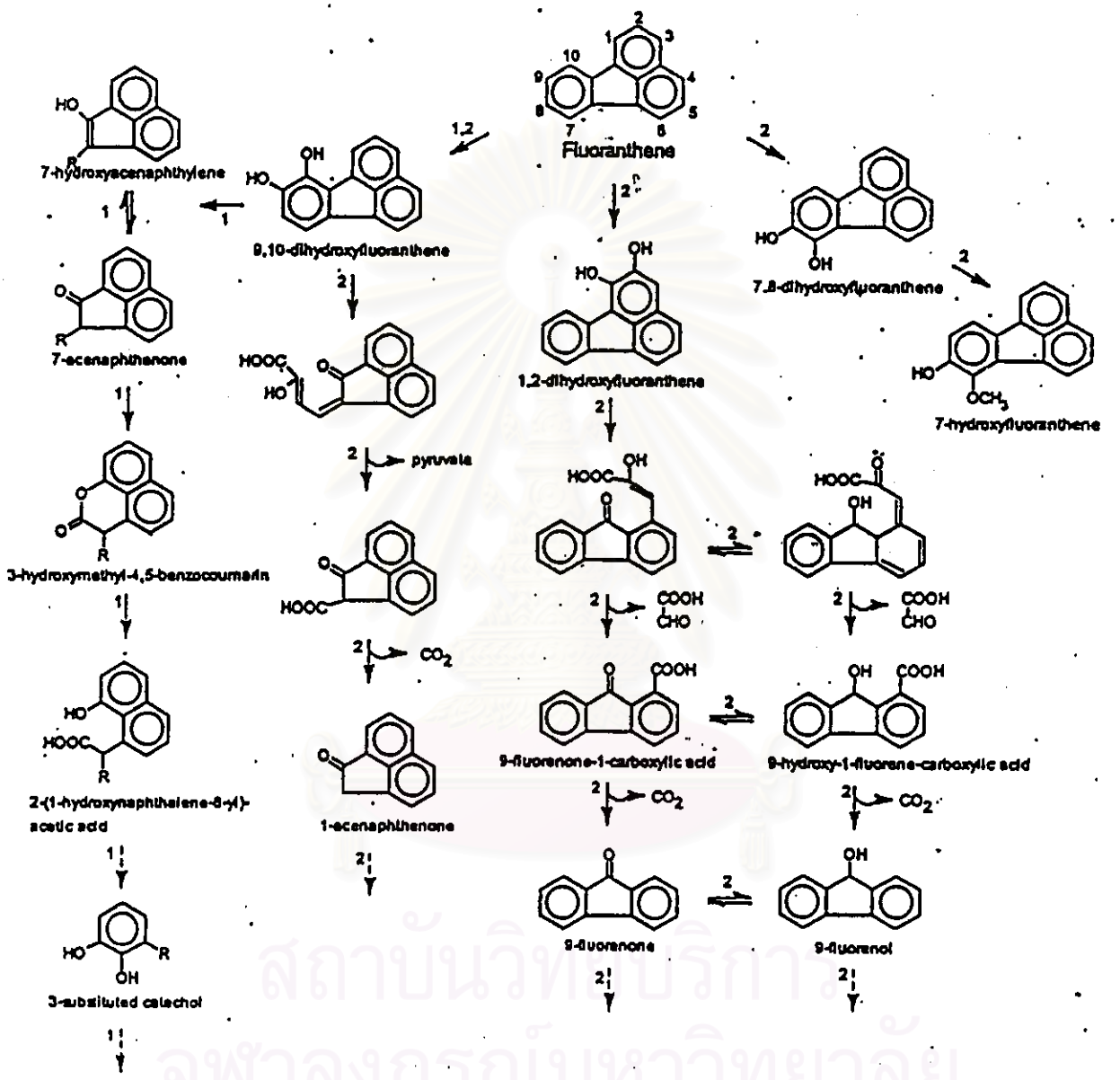

1. *Alcaligenes denitrificans* WW1 (Weissenfels *et al.*, 1990a; 1991)

2. *Mycobacterium* sp. PRY-1 (Kelley *et al.*, 1991; 1993)

Figure 2.7 Pathway of fluoranthene metabolism by bacteria

2.4 Enzymes of PAHs metabolism

Enzymes involved in PAHs metabolism, in particular naphthalene metabolism in *Pseudomonas* sp. have been extensively studied. In order to understand other PAHs metabolisms which employ these enzymes to catalyze the reaction, the detail of enzymes in naphthalene catabolism will be exemplified and discussed as follow:

2.4.1 Multicomponent aromatic ring dioxygenase

The initial step in the aerobic microbial degradation of aromatic compound is normally start by inducing two hydroxyl groups into the benzene ring via the action of enzyme dioxygenase (Butler and Mason, 1997), thereby forming a *cis*-dihydrodiols. This reaction would them followed by fission reaction and catabolism.

All of the PAH dioxygenases reported are multicomponent enzymatic system consisting of flavoprotein (ferredoxin reductase), ferredoxin and terminal oxygenase (Iron sulfur protein, ISP) (Ensley and Gibson, 1983; Haigler and Gibson, 1990a,b).

In case of naphthalene dioxygenase (Figure 2.7), ferredoxin$_{NAP}$ reductase could serve as NADH oxidoreductase. This enzyme functions as the initial electron acceptor which shuttles electrons from NADH to ferredoxin$_{NAP}$. Ferredoxin$_{NAP}$ functions as intermediate electron transfer protein in naphthalene dioxygenase system. Both ferredoxin$_{NAP}$ reductase and ferredoxin$_{NAP}$ are red iron-sulfur flavoprotein with 2 g-atoms each of iron and acid-labile sulfur per mole [2Fe-2S]. They possess molecular weight of 36,300 and 13,600, respectively (Sutherland *et al.*, 1995). Phenanthrene dioxygenase isolated from *Burkholderia* sp. RP007 revealed that ferredoxin reductase and ferredoxin possess molecular weight of 35,600 and 11,500, respectively (Laurie and Lloyd-Jones, 1999b).

ISP$_{NAP}$, the terminal oxygenase binding to naphthalene, requires both ferredoxin$_{NAP}$ reductase and ferredoxin$_{NAP}$ for its activity. ISP$_{NAP}$ catalyzes the reaction that brings two oxygen atoms to one of the aromatic ring of naphthalene in the presence of NADH ferredoxin reductase and ferredoxin. This protein has molecular weight of

158,000, including two subunits with molecular weight of 55,000 (α or large subunit) and molecular weight of 20,000 (β or small subunit). It also has 2[2Fe-2S] per mole.

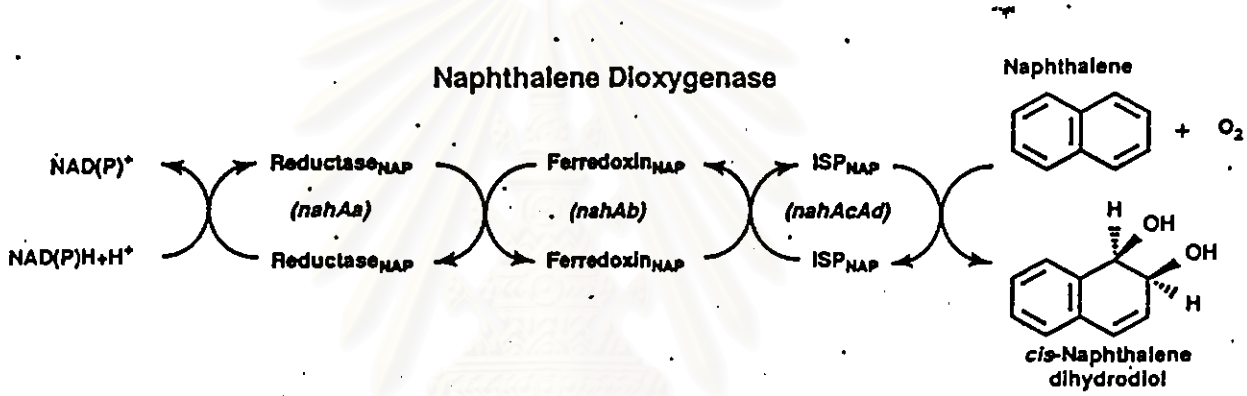

Figure 2.8 Oxidation of naphthalene to *cis*- naphthalene dihydrodiol by naphthalene dioxygenase multienzyme complex (Simon *et al.*, 1993)

Electrons from NAD(P)H are transferred to ISP_{NAP} by $reductase_{NAP}$ and $ferredoxin_{NAP}$. Then ISP_{NAP} catalyzes the addition of two oxygen atoms and two hydrogen redicals to the aromatic nucleus to form *cis*-naphthalene dihydrodiol (Simon *et al.*, 1993).

Ensley *et al.* (1983) studied the expression of naphthalene oxidation genes in *Escherichia coli* which eventually lead to the formation of indigo colour from indole as a substrate (Figure 2.9). The result suggested that indigo formation could be catalyzed by various aromatic hydrocarbon dioxygenases that made this reaction a valuable tool for the detection of this class of enzyme.

In recombinant strain of *Escherichia coli*, indole is formed from tryptophan by tryptophanase, a natural enzyme in *Escherichia coli*. Naphthalene dioxygenase formed by the expression of the cloned *Pseudomonas* DNA could oxidize indole to indigo. Cis-2,3-Dihydroxy-2,3-dihydroindole and indoxyl have not yet been isolated, their inclusion is based on the known activities of aromatic hydrocarbon dioxygenases and established mechanisms for the chemical synthesis of indigo (Ensley *et al.*,1983).

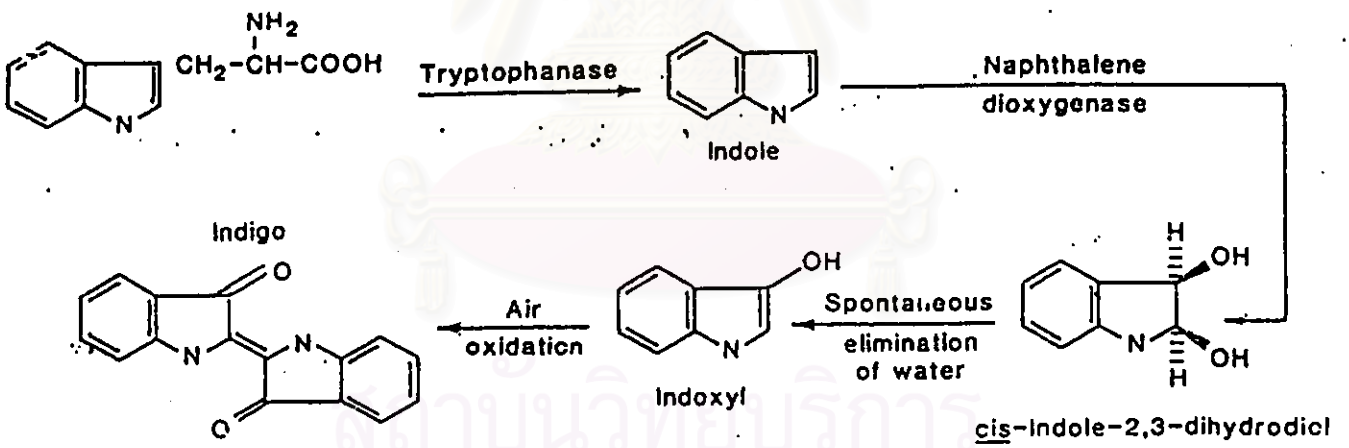

Figure 2.9 Proposed pathway of indigo formation in *Escherichia coli* recombinant strain (Ensley *et al.*,1983).

2.4.2 Dehydrogenase

Dihydrodiol dehydrogenase catalyzes the oxidation of the *cis*-dihydrodiol to dihydroxyaromatic compound. This enzyme requires NAD^+ as an electron acceptor. Patel and Gibson (1974) reported that naphthalene dihydrodiol dehydrogenase also capable of oxidizing *cis*-dihydrodiols of anthracene, phenanthrene, biphenyl, toluene, ethylbenzene and benzene.

2.4.3 Extradiol dioxygenase

Extradiol enzymes are responsible for the fission of the aromatic nucleus. Substrates of ring-cleavage dioxygenase usually contain hydroxyl groups on adjacent aromatic carbons. Enzymes cleave the ring between one hydroxylated carbon and its adjacent nonhydroxylated carbon (Eltis and Bolin, 1996). These enzymes have broad substrate specificity, since dihydroxynaphthalene dioxygenase can also used 3- and 4-methylcatechol as substrates (Sutherland *et al.*, 1995). In addition, 2,3-dihydroxybiphenyl can be served as substrate for aromatic extradiol dioxygenase (Kimura *et al.*, 1996).

2.3.4 Other enzymes

Other enzymes involved in naphthalene metabolism are;

Isomerase is an enzyme in *Pseudomonas* sp. which catalyze the conversion of 2-hydroxychromene-2-carboxylic acid to *o*-hydroxybenzylidinepyruvic acid (Eaton and Chapman, 1992).

Hydratase-aldolase converts the *o*-hydroxybenzylidinepyruvic acid to salicylaldehyde with the loss of pyruvate (Eaton and Chapman, 1992).

Salicylaldehyde dehydrogenase converts salicylaldehyde to salicylate. The action requires NAD^+ for activity (Sutherland *et al.*, 1995).

These enzymes could also be employed by bacteria to catabolize other PAHs and their metabolites. For example, initial dioxygenase, dehydrogenase, extradiol dioxygenase, isomerase, hydratase-aldolase and dehydrogenase catalyze each step of phenanthrene and anthracene metabolisms (Kiyohare *et al.*, 1994) (Figure 2.11). In addition, the finding of metabolites formed from pyrene and fluoranthene degradations (Figures 2.6 and 2.7) suggested that these enzymes also involved in pyrene and fluoranthene degradations (Cemiglia, 1992).

2.5 Genetics of bacterial PAHs metabolism

2.5.1 Genetics of naphthalene metabolism

Of all genetic studies on PAH degradation, the genetic of naphthalene degradation has been most extensively studied. The catabolism of naphthalene by Pseudomonads is often plasmid encoded as originally reported by Dunn and Gunsalus (1973). Further studies by using shot gun cloning, transposon mutagenesis, and also biochemical approach have demonstrated that the *nah* genes are organized into two 10 kb operons on plasmid NAH7 (83 kb) from *Pseudomonas putida* G7 (Yen and Gunsalus, 1982). The *nah* operon *nahAaAbAcAdBFCDE* encodes genes for the conversion of naphthalene to salicylate (upper pathway) (Figures 2.4) while *sal* operon encodes the genes responsible for the conversion of salicylate to central metabolites (lower pathway) *nahGHINLJK* (Yen and Gunsalus, 1982; Eaton and Chapman, 1992). A single regulatory gene *nahR* is located between the two operons and involved in the regulation of both (Schell and Sulordhman, 1989) (Figure 2.10). The genes for individual component of multicomponent naphthalene dioxygenase which concerts naphthalene to *cis*-naphthalenedihydrodiol have been designated *nahAa* (reductase$_{NAP}$), *nahAb* (ferredoxin$_{NAP}$), *nahAc* and *nahAd* (iron sulfur protein large and small subunit, ISPα, ISPβ), respectively (Figure 2.8) (Simon *et al.*, 1993).

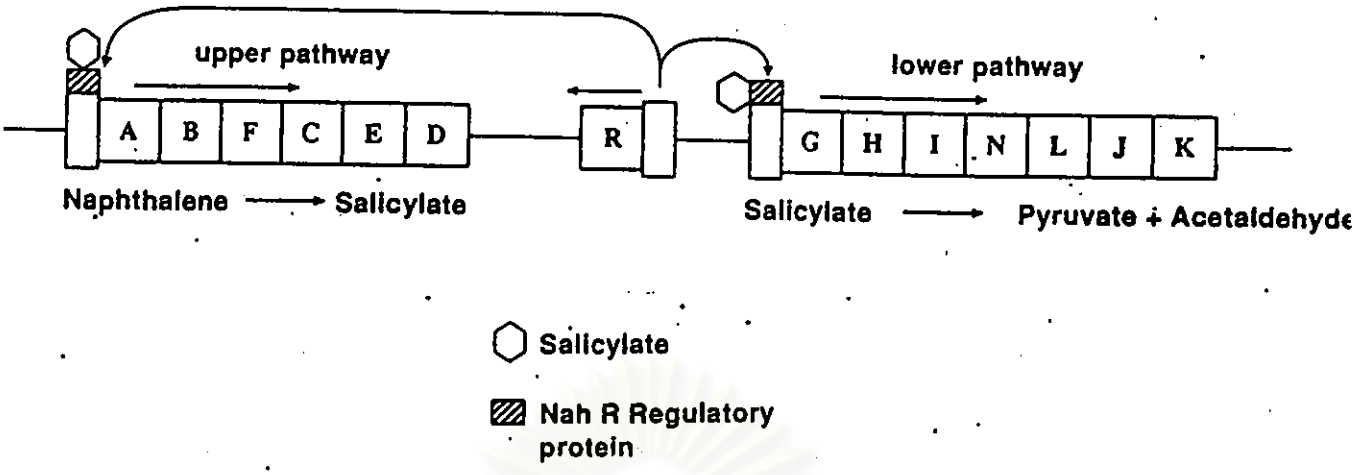

Figure 2.10 Naphthalene catabolic gene organization and regulation (Source: Sutherland *et al.*, 1995)

In *P. putida* strain NCIB9816 and NCIB9816-4, two operons of the dissimilatory genes (*ndo* genes and *nah* genes) are located on NAH plasmid-pWW60-1 (87 kb) and pDTG1, respectively (Cane and Williams, 1986; Platt *et al.*, 1995; Simon *et al.*, 1993). The genes responsible for naphthalene dioxygenase were cloned from *P. putida* NCIB9816 based on their ability to oxidize indole to indigo in *E. coli*. Total *P. putida* DNA was digested partially with *Sau*3A, the 10-20 kb fragments were ligated into *Bam*HI-digested pBR322 and the library was transformed into *E. coli* HB101. Recombinant plasmid pSKH300 containing a 14-kb insert was isolated then subcloned into pUC18 and pUC19. The functional region located to within a 2.7-kb *Eco*RI-fragment (Plasmid pSKH302) was sequenced and three successive open reading frames; *ndoA* (coding ferredoxin), *ndoB* (ISPα) and *ndoC* (ISPβ) were found with sizes 315, 1,351 and 584 bp, respectively (Kurkela *et al.*, 1988).

Furthermore, the *dox* gene cluster (9.8 kb) encoding dibenzothiophene-degrading enzymes from plasmid of *Pseudomonas* sp. C18 was found to be involved in metabolism of naphthalene to salicylic acid. Comparison of nucleotide sequneces revealed that *doxABC* are homologous to the *ndoABC* that encode naphthalene dioxygenase of *P. putida* (Denome *et al.*, 1993).

Other NAH-like plasmids pKA1, pKA2 pKA3 could be isolated from *Pseudomonas* sp. 5R, DFC49 and DFC50, respectively (Sanseverino *et al.*, 1993; Menn *et al.*, 1993). Whereas a 63 kb plasmid pLP6a from *Pseudomonas fluorescens* LP6a carries genes encoding enzymes with broad substrate specificity involved in the degradation of naphthalene, phenanthrene and anthracene. This plasmid also hybridizes to the plasmid NAH7 (Foght and Westlake, 1996).

Recently, the *nah* genes from *Pseudomonas stutzeri* AN 10 were found on chromosome. The *nahAaAbAcAdBFCED* (11,514 bp) cluster was characterized to be involved in the naphthalene degradation upper pathway (Bosch *et al.*, 1999).

2.5.2 Genetics of phenanthrene metabolism

The genes involved in phenanthrene metabolism have been established. Phenanthrene degradation in *Alcaligenes faecalis* AFK2 (Kiyohara *et al.*, 1990), *Mycobacterium* sp. (Guerin and Jones, 1988) and *Micrococcus* sp. S5P (Ghosh and Mishra, 1983) were found plasmid encoded.

Recently, genes encoding phenanthrene-degradation enzymes (*pah* genes) have been more extensively studied. Kiyohara *et al.* (1994) could clone and characterize chromosomal *pah* gene cluster which encodes enzymes for the upper pathway of phenanthrene and naphthalene from *P. putida* OUS82. In that experiment, partial *Sal*I digests of the total DNA from OUS82 were ligated at the *Sal*I site of the tetracycline resistance gene of pSTK10, a cosmid constructed by insertion of a 1.5-kb *Hin*dIII digest carrying the kanamycin-resistant gene from pUC4K into the *Eco*RI site of cosmid vector pLAFR1, then packaged in vitro and transfected into *E. coli* HB101. The 17 blue indigo-forming colonies on LB plates were found from 200 tetracycline-sensitive and kanamycin-resistant HB101 clones. Characterization of cloned genes (*pah*) suggested that they were classified in *nah*-like gene group (Kiyohara *et al.*, 1994). In addition, *pahA* gene which encodes dioxygenase was identified and sequenced. The results showed that *pahA* consisted of four cistrons, *pahAc, pahAb, pahAc and pahAd* which encode ferredoxin reductase, ferredoxin and two subunits of iron-sultur protein, respectively (Takizawa *et al.*, 1994). Due to ability of utilizing wide variety of PAHs of *P.*

putida OUS82, possible relationship between PAH degradation pathways was postulated (Figure 2.11) (Kiyohara *et al.*, 1994).

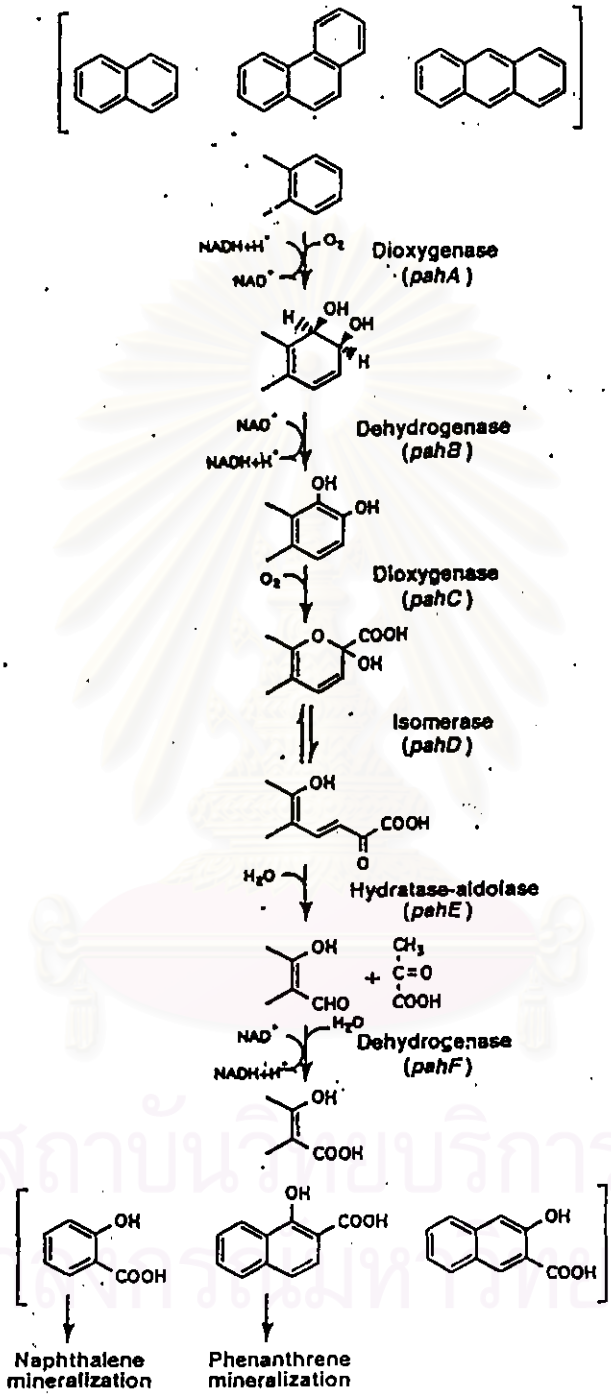

Figure 2.11 The postulated upper pathway for naphthalene, phenanthrene and anthracene in *P. putida* OUS82 (Kiyohara *et al.*, 1994)

Two gene clusters, *phdEFABGHCD* (8.5 kb) and *phdIJK*, are found encoding enzymes responsible for the oxidation of phenanthrene to 1-hydroxy-2-naphthoic acid and the latter compound to o-phthalate in *Nocardioides* sp. K P7, respectively (Iwabushi and Harayama, 1997; 1998a; 1998b; Saito *et al.*, 1999). The deduced *phd* products exhibited moderate degrees of homology with isofunctional enzymes found in other aromatic compounds degradation pathways; PhdA, PhdB, PhdC, PhdD showed significant homology to ISPα, ISPβ, ferredoxin and ferredoxin reductase, respectively. Meanwhile, the *phdC* gene product which was the [3Fe-4S] type ferredoxin has never been reported as ring-hydroxylating dioxygenase (Saito *et al.*, 1999).

Furthermore, genes involving in phenanthrene degrading from *Comamonas testosteroni* GZ39 and *Burkholderia* sp. RP007 (*phn* genes) showed no or very low homology with the *nah*-like gene group based on hybridization and PCR with probe and primers corresponding to *nah* gene, even both strain can utilize naphthalene (Goyal and Zylstra, 1996; Laurie and Lloyd-Jones, 1999b).

The genes responsible for the upper pathway of phenanthrene degradation in *Burkholderia* sp. RP007 was cloned and the *phnSFECDAcAdB* gene cluster encoding enzymes (upper pathway) was determined (Laurie and Lloyd-Jones, 1999a). These genes are significantly different in sequence and gene order from the previously characterized gene for PAH degradation (Laurie and Lloyd-Jones, 1999b).

The *meta*-clevage genes from *Burkholderia* sp. RP007 were also obtained by screening for catechol *meta*-clevage activity after shot gun cloning (Laurie and Lloyd-Jones, 1999a).

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2.6 PAHs in Thailand

In Thailand, numerous reports indicated PAHs contamination in several areas.

Panther *et al.* (1996) could detect twenty PAHs, including these sixteen US-EPS priority compounds as well as benzo(e)pyrene, perylene, coronene and anthranthrene from airborne samples taken at Chulalongkom University, Bangkok, Thailand during April 1993 to April 1994. Figure 2.12 depicts the annual average concentration of PAHs detected.

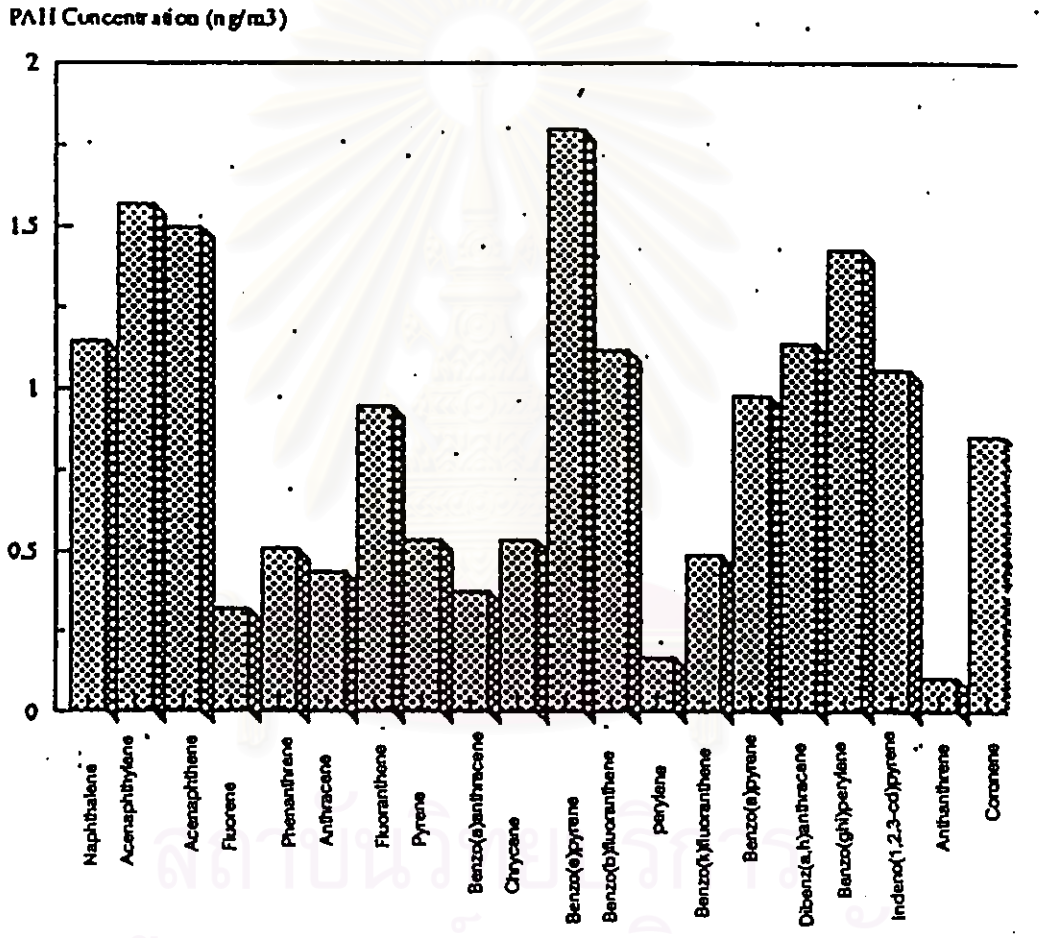

Figure 2.12 The annual average concentration of each individual PAH in Bangkok (Panther *et al.*, 1996)

In other study, Amagai *et al.* (1999) revealed the presence of sixteen PAHs with 4 to 7 rings in soil from the city of Chiang-Mai, Thailand in February 1996, among these pyrene and fluoranthene were found to be the highest amount as shown in Table 2.3.

Table 2.4 PAHs concentrations in soil in Chiang-Mai, Thailand (Amagai *et al.*, 1999)

PAH	Molecular weight	Arithmetic Mean	±	SD	Geometric Mean	SD	Max	Min	Max/min
Fluoranthene	202.3	146	±	75	131	1.62	418	37.1	11
Pyrene	202.3	168	±	103	142	1.82	526	39.7	13
Benz(a)anthracene	228.3	35.0	±	20.2	30.3	1.71	91.9	11.7	7.9
Chrysene	228.3	61.6	±	29.1	56.2	1.53	156	26.5	5.9
Benzo(e)pyrene	252.3	43.8	±	23.1	38.3	1.72	114	11.6	10
Benzo(b)fluoranthene	252.3	46.4	±	23.0	41.2	1.68	122	10.0	12
Benzo(k)fluoranthene	252.3	10.6	±	6.0	9.10	1.78	31.3	2.12	15
Benzo(a)pyrene	252.3	22.4	±	10.4	20.2	1.62	53.6	6.81	7.9
Benzo(ghi)perylene	276.3	97.7	±	47.8	86.1	1.73	251	17.8	14
Indeno(1,2,3-cd)pyrene	276.3	39.0	±	19.7	33.7	1.82	101	5.07	20
Dibenz(a,h)anthracene	278.4	5.21	±	2.44	4.67	1.64	12.9	1.27	10
Picene	278.4	9.22	±	4.96	8.26	1.60	28.6	2.27	13
Dibenz(a,c)anthracene	278.4	9.05	±	5.19	7.86	1.72	26.6	1.97	13
Benzo(b)chrysene	278.4	5.79	±	2.50	5.27	1.58	13.0	1.87	6.9
Coronene	300.4	93.8	±	46.4	80.9	1.83	202	14.1	14
Debenzo(a,e)pyrene	302.4	30.6	±	13.8	27.7	1.59	68.6	9.67	7.1
Total PAH		824	±	399	738	1.63	2196	205	11

n=30

All concentration in ng/g soil.

Total concentrations of 16 PAHs in soil at different sampling sites (shown in Figure 2.13) suggested that PAHs concentrations were highest on the roadside with heavy traffic, indicating that vehicles are the main determinants of PAHs accumulation in soil in Chiang-Mai.

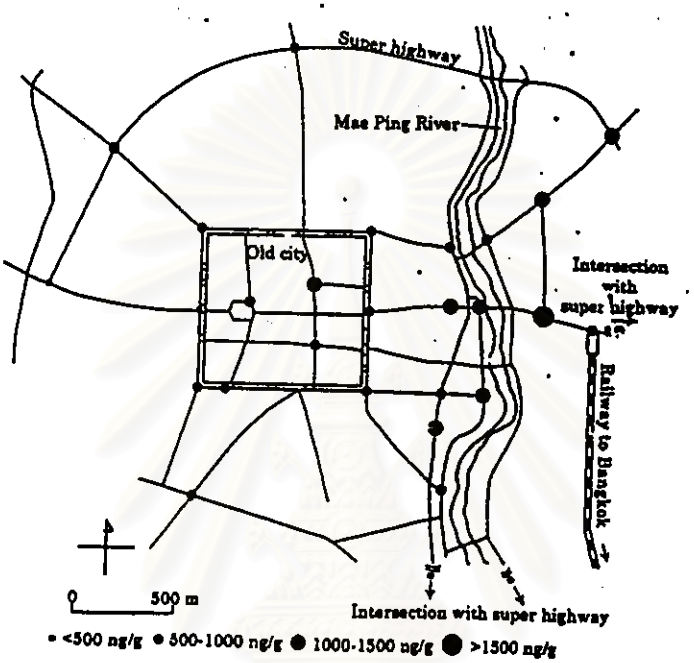

Figure 2.13 Total concentrations of 16 PAHs in soil at different sampling sites in the city of Chiang-Mai, Thailand (Amagai *et al.*, 1999).

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