CHAPTER I

G E N E R A L I N T R O D U C T I O N

GENERAL INTRODUCTION

1.1. CHEMICAL CARCINOGENESIS: GENERAL

It has been a constant human endeavour to understand the cause and effect relationship for any disease and cancer is no exception. Cancer arises due to exposure of the organism to the causative agent and the response of the organism to this carcinogenic insult. Epidemiological studies correlated certain types of cancer to one or many of the following factors viz., country or area of residence, consumption of particular diet, working with particular chemicals, following a special social custom, etc. Thus nearly 80-90% of human cancer can be linked to environmental causative agents (Heidelberger 1975). Radiation has been linked to certain occupational cancers observed among radiologists, uranium miners or farmers and sailors, while viruses as a cause of human cancer is not unequivocally established. Environmental chemicals are implicated in bulk of the human cancers (Doll 1977). These chemicals may be naturally occurring in our diet or environment or may be man-made. The list of chemicals known (or suspected) to be carcinogenic for man has a number of chemical mixtures (certain tars, soots, oils, betel nut extracts and cigarette smoke) or pure chemicals such as aromatic amines, aromatic nitro compounds, alkylating agents, aliphatic halide,

synthetic estrogen and several inorganic chemicals. Also included are chemicals which are highly suspected to be human carcinogens, e.g., certain polycyclic aromatic hydrocarbons, several aromatic amine derivatives, some aliphatic nitrosamines and nitrosamides and fungal toxins (Miller and Miller 1977). Polycyclic aromatic hydrocarbons (PAHs) are present in soot which was suspected to be human carcinogen two hundred years back and are also components of coal tar for which experimental carcinogenesis has been proved for nearly 70 years. Interaction of such chemicals like PAHs with other factors such as diet and in conjunction with factors beyond human control (at present) like genetic susceptibility may decide the occurrence of cancer in human population.

1.2. OCCURRENCE OF PAHs:

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants. They are present in industrial and automobile exhausts, air, cigarette smoke, soot, coal tar and pitch. In soil and water, they arise mainly from air pollution. The ubiquitous existence of PAHs are amply demonstrated by the endless list of food items which contain PAHs. They are believed to arise predominantly through inefficient cumbustion of fossil fuels and other organic matter. Industrially, PAHs are synthesized

at high temperatures (1000 - 1600°) in absence of sufficient oxygen, at which organic compounds breakdown by cracking, with release of C, H and CH radicals. These radicals polymerize via "nascent acetylene" to form a variety of PAHs. They are also obtained by pyrolysis (500 - 600°) of biological compounds like carbohydrates, amino acids and fatty acids. Tobacco, stigmasterol, aliphatic hydrocarbons, agar-agar, natural dyes, humectants, glues, starches and log wood also produce PAH on pyrolysis. The different PAHs present in these sources are benz(a)anthracene, benzo(a)pyrene (BP), benzo(e)pyrene (BeP), dibenz(a,h)anthracene (DBA), dibenzopyrene, chrysene, benzofluoranthenes etc. (Fig. 1.1). Among these PAHs to which human population is chronically exposed are highly potent carcinogens like BP and DBA. It is likely therefore that such PAH may be responsible for a significant percentage of human cancer (IARC 1973).

The danger of exposure to such PAH is highlighted by the fact that BP is found in a host of commonly encountered items. BP has been detected in broiled, smoked and barbecued meat, sausage, ham, fish and steaks, in fruits and vegetables, cereals, vegetable oils, roasted coffee, tea, baker's yeast and whisky. Leaves of different trees, furnace blacks, automobile tyres and rubber stoppers also have BP (IARC 1973). Foods can get contaminated with pyrolytically generated PAH or by contact with either petroleum

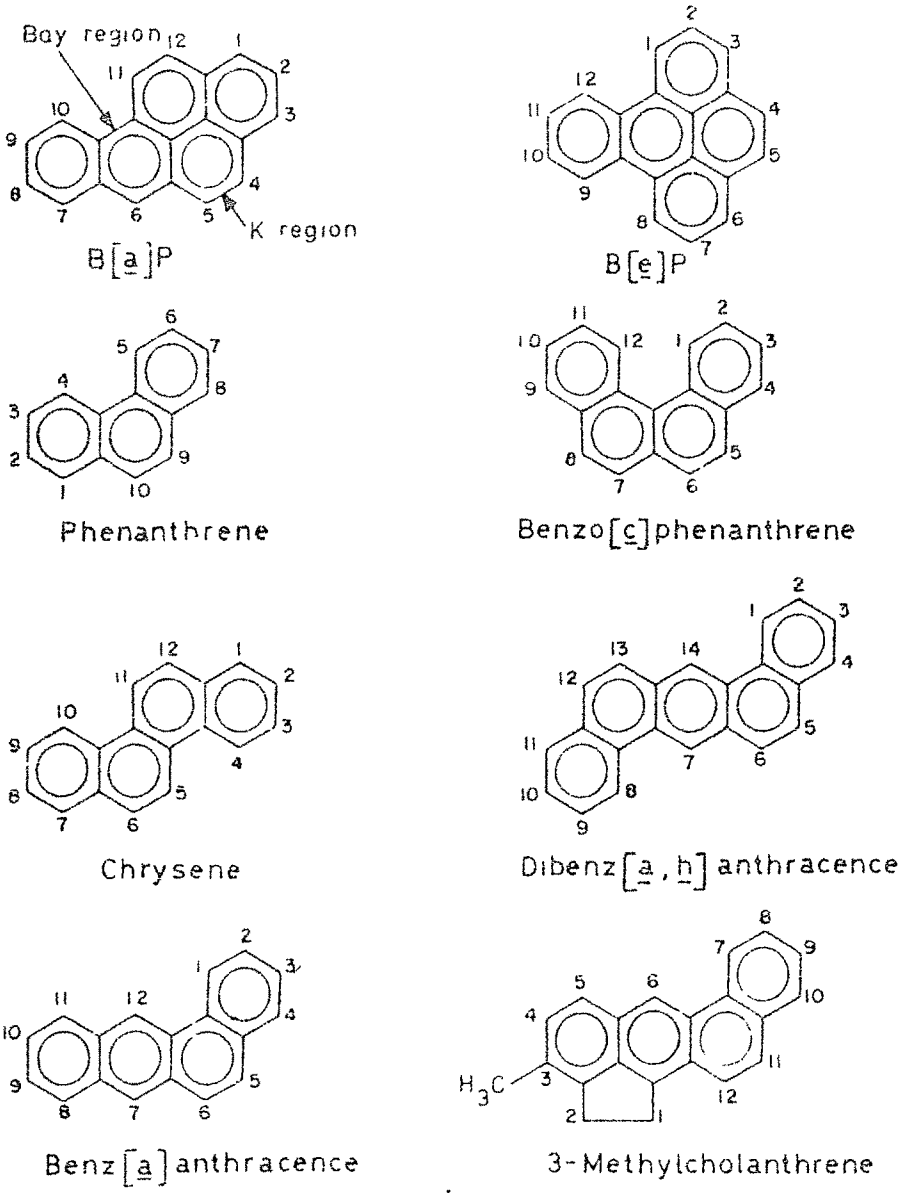


FIG. 1.1. Structures of some polycyclic aromatic hydrocarbons. (Thakker et al. 1985).

products and coal tar products or from deposition of air pollution particulates inside the foods (Dunn 1982). Biosynthesis of PAH by plants, algae or microorganisms has been suggested in the past (IARC 1973), but this could be alternatively explained as contamination of PAH from non-biosynthetic routes (Dunn 1982). The amount of PAH consumed by human beings therefore will vary with the type of diet, smoking habit, place of residence, occupation and many other factors. Hatch et al. (1986) gave an estimate of intake of PAH in the range of 1.2 mg per year per person and listed the contributory factors in the following order: grains (40%) > oils and fats (25%) > leafy vegetables = smoked meats = fruits > grilled foods (charcoal fuels). BP represents few percent of total PAH and BP intake by human beings is estimated at about 0.1 µg per kg per day (Preussman 1976). Transfer of PAH from diet to milk as shown for few mammals - rat, rabbit and sheep (West et al. 1976) highlights the dangers of PAH-exposure to suckling infants.

1.3. BIOLOGICAL ACTIVITIES OF PAHs:

a) Carcinogenicity:

Soot was first noted as a cause of scrotal cancer in chimney sweeps in 1775 by Sir Percival Pott (IARC 1973). Coal tar was shown to be carcinogenic to rabbits and mice in

early parts of twentieth century and PAH was implicated as the main carcinogenic component of coal tar (Thakker et al. 1985). DBA, a pure pentacyclic PAH was shown by Hieger in 1930, to cause cancer in experimental animals (Thakker et al. 1985). Employing fluorescence spectroscopy, the same group identified many carcinogenic constituents of coal tar including BP (Cook et al. 1932; 1933). BP has produced tumors in a host of species at a variety of target organs in response to different routes of administration. Mouse, rat, hamster, guinea pig, rabbit, duck, monkey and newt developed tumors in response to BP administration by one or many of the different routes viz. oral, skin application, inhalation/intratracheal, subcutaneous/intramuscular, intraperitoneal, intravenous, intrabronchial, transplacental, lung fixation, implantation in various organs (stomach wall, renal parenchyma, brain etc.), injection into renal pelvis, vaginal paintings etc. Target organs for different routes of BP-administration include skin, mammary gland, lung, liver, forestomach, oesophagus, brain, colon etc. (IARC 1973). In cell culture, mouse fibroblasts were malignantly transformed by BP (Marquardt et al. 1976).

Very few reports of carcinogenic effects of BP on man are available. Regressing verrucae developed on daily application of 1% BP solution to 26 patients for 4 months. Squamous epithelioma nodule was observed in man accidentally exposed to BP for 3 weeks (IARC 1973).

BeP on the other hand was shown to be weakly carcinogenic or noncarcinogenic in various experimental systems (IARC 1983).

b) Toxicity and other effects:

Toxic effects of BP has been studied in several systems. LD_{50} for mouse was shown to be 250 mg kg^{-1} (i.p.), ID_{50} (skin-irritation) for mouse was 5.6 x 10^{-5} mmol per ear. BP induced liver damage in mice. It was shown to be embryotoxic and teratogenic in mice. Single i.p. dose of 10 mg kg^{-1} decreased growth rate in young rats. BP induced destruction of all cell types in human bronchial mucosa cell cultures (IARC 1983). It also induced infertility in mice (Mackenzie and Angevine 1981). BP was shown to accumulate in gonads of flatfish and subsequent egg development was shown to be abnormal (Hose et al. 1981).

Blanton et al. (1986) demonstrated inhibition of antibody response by BP but not by BeP in mice and murine cells. BP but not BeP was shown to suppress antibody production by mouse spleen cells in vitro and this was not affected by addition of metabolizing enzymes (White and Holsapple 1984). Interferon production was inhibited by BP in vivo (Griffin et al. 1986) and BP and chrysotile fibres inhibited induction of viral interferon (Hahon and Booth 1986). Interleukin-1 production in vitro by macrophages

was increased by BP, which might explain suppression of immune response (Lyte and Bick 1986).

1.4. METABOLISM OF PAHs:

1.4.1. General:

Most mutagenic or carcinogenic PAHs are usually hydrophobic chemicals that are metabolized by enzyme systems localized mainly in the liver. The metabolism can be divided into 2 phases. During phase I, one or more polar groups (e.g. -OH) are introduced into the hydrophobic molecule which then becomes substrate for phase II-conjugating enzymes, followed by excretion of the polar product(s) from the cell, organ or body (Singer and Grunberger 1983). Although this metabolism is for the purpose of detoxification and removal of xenobiotic, it has harmful side-effects. In some cases, this leads to formation of more toxic metabolites (Guengerich and Liebler 1985) or more potent carcinogenic products (Singer and Grunberger 1983). Millers were the first to postulate that most carcinogens are metabolically activated to proximate and ultimate carcinogens and it is the latter, a strongly electrophilic moiety that combines with cellular macromolecules (Miller 1970; Miller and Miller 1977).

The most important Phase I enzyme system is a group of enzymes called mixed function oxidases (MFO) or

monooxygenases (MO), located principally in endoplasmic reticulum of the cell (microsomal fraction). For PAH, the reaction by these enzymes are hydroxylations and therefore they are called aryl hydrocarbon hydro(xy)lases (AHHs). AHH is ubiquitous enzyme system observed in 90% of tissues examined. Lung, liver, gastrointestinal tract and kidney in rat, mouse, hamster and monkey are particularly rich in these enzyme. It has also been detected in human liver, lung, placenta, lymphocytes, monocytes and alveolar macrophages. It is present mainly in microsomes, but is also present in nuclei, nucleoli and mitochondrial fractions of the cell (Singer and Grunberger 1983).

AHH is a multicomponent system with at least two proteins: cytochrome P-450 (a family of different cytochromes) and NADPH-cytochrome C (or P-450) reductase, and a phospholipid : phosphatidylcholine. AHH also needs NADPH as cofactor and molecular oxygen for its function. Cytochrome P-450 is a family of hemoprotein which facilitates the transfer of one atom of molecular oxygen to substrate, while other atom is reduced to water in presence of NADPH.

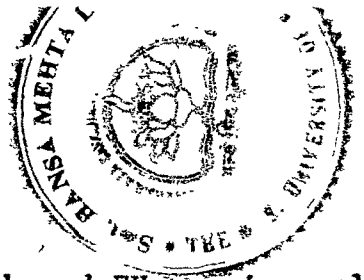
$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

Using genetic, electrophoretic and immunological techniques, multiple forms of cytochromes P-450 have been charcterized and it was recommended to use "Polysubstrate monooxygenase" as general name and P-450 as trivial name for these isozymes.

P-450 are inducible and that explains increase in activity of AHH after exposure to certain chemicals in vivo and in culture. Different inducers stimulate different P-450 isozyme. In rat liver, out of 5-7 forms of P-450, phenobarbital (PB) induces P-450a and P-450b. While 3-methylcholanthrene (MC), a PAH, induces P-450a and P-450c (also called P-448). Aroclor, a polychlorinated biphenyl, induces a,b and c forms of P-450. Isosafrole treatment induces P-450d. Similarly for rabbit liver and lung and for mice and human liver, P-450 isozymes have been characterized. (Singer and Grunberger 1983).

The e^- transfer from NADPH to P-450 is facilitated by the flavoprotein enzyme NADPH cytochrome C (or P-450)-reductase. It has one molecule each of FMN and FAD per polypeptide. The N-terminal hydrophobic region of this enzyme binds to membrane and interacts with P-450. Unlike P-450, there is only one form of this enzyme (Singer and Grunberger 1983).

PAHs when metabolized by AHH, produce very reactive epoxides which either isomerize spontaneously to form quinones and monohydroxy derivatives or are further metabolized by epoxide hydrolase (EH), also called epoxide hydra(ta)se (Thakker et al. 1985). EH is present in all organs and tissues of mammals. EH is present mainly in microsomes but is also present in nuclear membrane, and in cytosol. The



microsomal and nuclear membrane bound EH are immunologically identical but are different from cytosolic enzyme. EH has large amount (56%) of hydrophobic residues, and is also inducible but its induction is different from induction of AHH. PB induces EH but MC does not, indicating genetic control of induction of EH away from Ah locus. Trans-stilbene oxide is an exclusive inducer of EH in liver of rats and hamsters (but not mice). There are suggestions of multiple forms of EH. Simple arene oxides like BP-4,5-oxide or styrene oxide are good substrates for EH but BP-diolepoxides are poor substrates (Singer and Grunberger 1983).

The phase II reactions are caried out by three major conjugation enzymes. Glutathione-S-transferase, a group of enzymes, which inactivates activated metabolites of PAH by conjugation with reduced glutathione, is located in microsomes as well as in cytosol. This can inactivate BP-7,8-epoxide, BP-7,8-dihydrodiol and BP-7,8-diol-9,10-epoxide. Most of the conjugates is excreted in bile and some of it undergoes mercapturic acid biosynthesis which later gets excreted in urine. Sulfotransferase is a cytosolic phase II enzyme which esterifies phenolic group of PAH, carbohydrate or steroid with sulfate. Prior to this transfer SO_4^{2-} is enzymatically activated in presence of ATP and Mg^{2+} to 3'-phosphoadenosine-5'-phosphosulfate (PAPS), which then transfers activated SO_4^{2-} group to phenolic group. All phenol

and quinone metabolites of BP are sulfated but dihydrodiols are poor substrates. The third conjugating enzyme of phase II is UDP-glucuronyl transferase (GT), which conjugates substrates with activated glucuronic acid. GT is a microsomal enzyme present in liver, lung, kidney, stomach and skin but not in brain and spleen. GT is inducible and MC-induced form of GT has high activity towards BP and its metabolites like 3-OH-BP or BP-3,6-quinone, but low activity towards BP-7,8-dihydrodiol. Simultaneous induction of AHH and GT in mouse in response to MC indicates genetic link in regulation of these two enzymes (Singer and Grunberger 1983).

BP and its metabolites are eliminated mainly through hepatobiliary system and gastrointestinal tract in different species independent of the route of administration (IARC 1973).

1.4.2. Metabolism of BP:

Metabolism of BP has been most extensively studied among all PAHs. Earlier studies identified several phenols, dihydrodiols and quinones of BP (Sims 1967; 1970; Waterfall and Sims 1972; Kinoshita et al. 1973). Application of high performance (pressure) liquid chromatogrphy (HPLC) from 1974 onwards, has greatly facilitated isolation and identification of not only major but also minor metabolites of BP (Selkirk et al. 1974). General pattern of HPLC-separation of BP-metabolites has been depicted by Singer and Grunberger (1983).

BP is generally converted to hydroxy, epoxy, diolepoxy and quinone derivatives. The general plan of metabolic enzymes and metabolites formed have been exhaustively reviewed by Singer and Grunberger (1983) and Thakker et al. (1985). In rats, BP metabolism has been shown to occur in several organs, their cells in culture and in subcellular fractions. Metabolism of BP has been studied for rats in liver (Conney et al. 1957; Sims 1967; 1970; Kimura et al. 1977; Ben-Itzhak et al. 1986), liver cells (Vadi et al. 1975), hepatoma cells (McManus et al. 1986), liver nuclei and microsomes (Lafarge-Frayssinet et al. 1981) and with purified liver microsomal enzymes (Wood et al. 1976). Metabolism of BP has been shown in rat lung homogenate (Kimura et al. 1977), lung culture (Cohen et al. 1976) and with rat intestinal microsomes (Stohs et al. 1976). BP-metabolism has also been demonstrated in fetal and placental tissues (Berry et al. 1977). Comparative metabolism in rat and human tissues have been carried out with lymphocytes (Selkirk et al. 1976; Gelboin et al. 1976) and lung culture (Cohen et al. 1976). Metabolism of BP in mouse, hamster, Rhesus monkey, fish and fungus has been carried out by various workers (Thakker et al. 1985). Among the unusual routes BP-metabolite formation is one through bacterium (Gibson et al. 1975) and by atmospheric epoxidation with O_3 (Pitts et al. 1980). Methylation of BP-metabolites and metabolism of methyl-BP have also been reported (Lombardi et al. 1981; Konieczny and Harvey 1982; Silverman 1981).

Metabolites formed in one cell can be translocated either out of the cell (Jones et al. 1978; Chipman et al. 1981; Merrick et al. 1985) or to another cell (Nordenskjold et al. 1981). BP-metabolites can also be transferred through placenta (Kihlstrom 1986). Some extraneous factors also affect the metabolism of BP e.g. physical state of the contaminant i.e. particulate or microcrystalline (Bevan and Manger 1985; Tornquist et al. 1985) and presence of other agents like polonium-210 an α-emitter (Little et al. 1978) or chrysotile fibres (Paterour et al. 1985) or nitrogen dioxide in air (Takeda and Teranishi 1986).

Individual microsomal metabolites formed from BP have been described in excellent details by Thakker et al. 1985. Brief account of the principal metabolites of BP is as follows. The major oxidative metabolites of BP formed by AHH and EH are shown in Fig. 1.2. Arene oxides are principal primary metabolites. K-region 4,5-oxide and non-K-region 2,3-, 7,8-, and 9,10-epoxide are also formed. The arene oxide can form phenol and quinone in absence of EH while it can form dihydrodiol in presence of EH. Enzymes exercise regio- and stereoselectivity throughout the metabolic steps like addition of epoxide group or conversion of epoxide to dihydrodiols. The dihydrodiols are then converted to diolepoxide responsible for BP-carcinogenesis. The pathways are depicted in Fig. 1.3. The nomenclature system

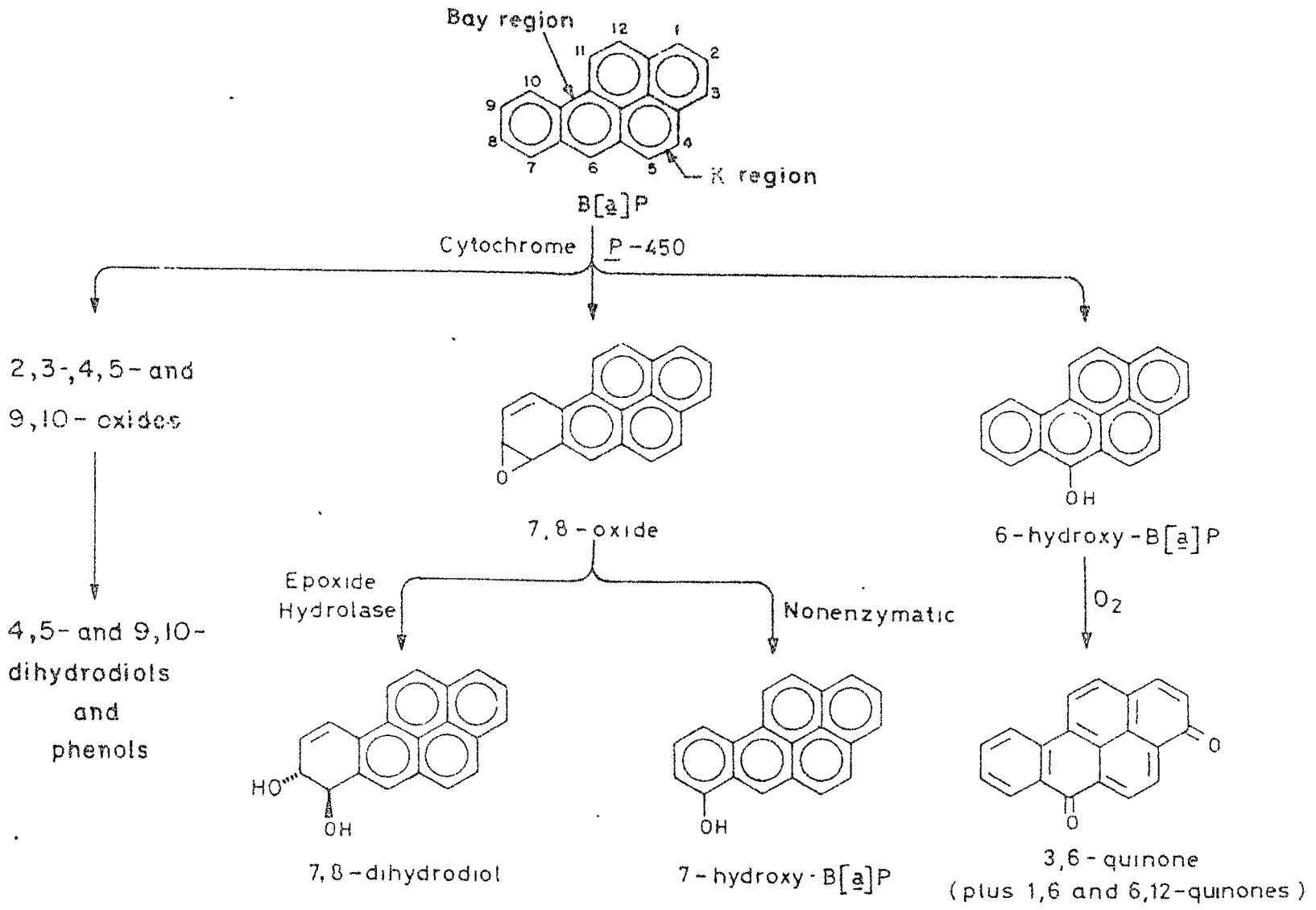


FIG. 1.2. Major oxidative metabolism of BP.

(Thakker et al. 1985).

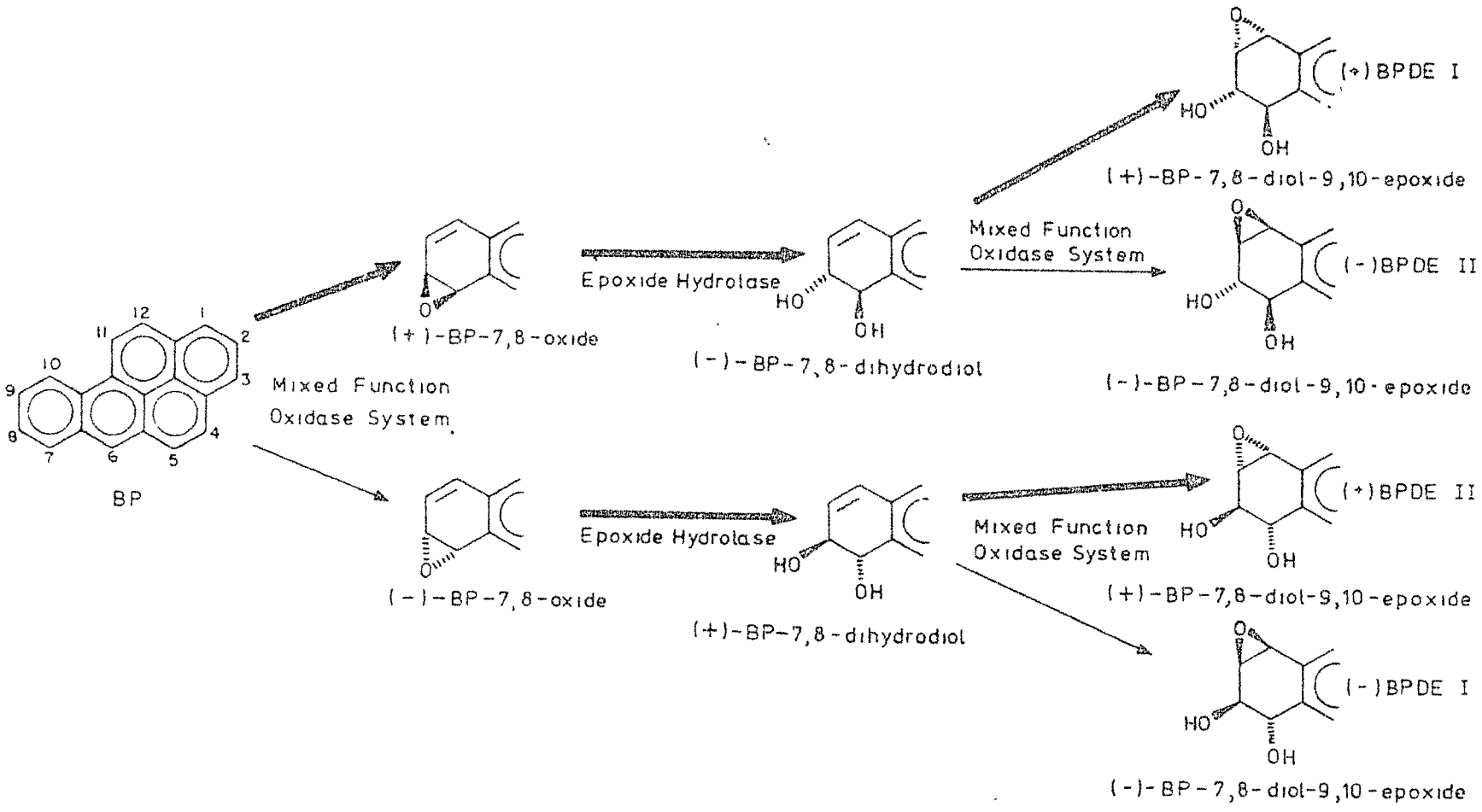


FIG. 1.3. Formation of BP-metabolites responsible for carcinogenicity. (Singer and Grunberger 1983).

followed for the isomers of BP-7,8-dihydrodiol-9,10-epoxide (BPDE) has been different with different groups. Gelboin and coworkers prefer to call 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydroBP as BPDE I and 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydroBP as BPDE II. However, same BPDE I is referred to as BPDE-2 by Jerina's group, and as anti-BPDE by Harvey's group. Correspondingly BPDE II is referred to as BPDE-1 and syn-BPDE. Another way to denote BPDE I structure is r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydro BP where r is reference substituent while c and t refer to positioning of oxygen function with respect to tetrahydro-benzene ring i.e. cis (same side) and trans (opposite side) (Phillips and Sims 1979). For the sake of uniformity, BPDE I and II nomenclature will be followed in the thesis. At each stage of metabolism arene oxide, dihydrodiol, or diolepoxides can be conjugated out of the mainstream of metabolism by detoxifying system.

The metabolic activation of BP to oxygenated metabolites, considered so far is by monooxygenase or two-electron oxidation pathway. However, a limited support is also available for one-electron oxidation pathway for PAH in general and BP in particular (Cavalieri and Rogan 1985). This pathway produces radical cations or radicals, depending on the molecule in which oxidation occurs. Removal of π-electron in an aromatic system generates radical cations while one n-electron oxidation of a phenol or amine with subsequent

loss of proton produces a radical. Importance of free radical formation (Selkirk 1980; Sullivan 1985) and radical cations (Cavalieri and Rogan 1985) in metabolism and subsequent effects have been considered by these authors. However, overwhelming amount of work and support is for the enzyme-mediated oxidative (2 electron) mechanism of activation.

1.4.3. Importance of bay region metabolism for carcinogenicity:

Pullmans (1955) attempted to correlate physicochemical properties of the PAH and its carcinogenicity using quantum chemical calculations of reactivity. However, since metabolism was not taken into account, these attempts met with limited success. With advent of metabolism, attempts were once again made to correlate quantum mechanical property of ground state and the ease of metabolism in that region (Shipman 1978). These calculations led to many possible sites at which metabolism can occur, principally K and L sites of PAH, thus leading to speculation regarding carcinogenicity due to K region metabolites (Harvey et al. 1975; Jerina and Daly 1974). However, numerous in vivo studies pointed towards importance of bay region metabolites of PAH in biological activity, hence Jerina and Conney's group proposed bay region epoxide theory of PAH-induced mutagenicity and carcinogenicity which was supported by others (Lehr et al. 1978; Harvey and Dunne 1978). Quantum chemicl calculations for metabolism and reactivity of bay region was made by Loew et al.(1978).

Model was also proposed to indicate blockage of K-region and redistribution of electronic charge of PAH on bonding with oxidative enzymes to facilitate formation of non K metabolites (Miertus and Majek 1981). Extended bay region theory was proposed in quantitative form to include areas around K-region (Yan 1985).

1.5. BIOLOGICAL ACTIVITIES OF THE METABOLITES OF BP:

a) Carcinogenicity:

The metabolites of BP show tumorigenicity to a varying degree in different tumor models viz. chronic studies, inhalation-promotion studies and newborn mouse. When arene oxides of BP (4,5-, 7,8-, 9,10- and 11,12-oxide) were tested on mouse skin (Levin et al. 1976) and in newborn mice (Wislocki et al.1978), BP-7,8-oxide was observed to be strongly tumorigenic. When monohydroxy derivatives were tested for skin tumorigenicity 2-OH-BP showed strong activity (Kapitulnik et al. 1976; Wislocki et al. 1977; Slaga et al. 1978). BP-7,8-dihydrodiol was more carcinogenic than BP in newborn mice (Kapitulnik et al.1977). In newborn mice (Kapitulnik et al. 1978b) and in skin-tumor initiation model (Levin et al. 1977) (-)isomer of trans-7,8-dihydrodiol BP was more carcinogenic than (+)isomer. Slaga et al. (1976) demonstrated carcinogenicity of the isomer BPDE I in skin tumor initiation model. Kapitulnik et al. (1978a)

differentiated among the diolepoxide isomers, that in newborn mice, BPDE I was tumorigenic while BPDE II was toxic. The (+)optical enantiomer of BPDE I was shown to be more tumorigenic than (-)enantiomer (Buening et al. 1978). Tables 1.1 and 1.2 summarize the results from two test systems. BPDE has also been shown to activate C-Ha-ras-1-proto-oncogene in vitro (Marshall et al. 1984).

b) Mutagenicity:

BP has been shown to be mutagenic in several test systems (IARC 1983). Metabolites of BP have been tested for mutagenicity in several test systems, most notably in reverse mutation-test of Ames (McCann et al. 1975) using histidine-dependent strains of Salmonella typhimurium and in mammalian forward mutation test using Chinese hamster V79 cells (Newbold and Brookes 1976). In absence of metabolic activation system, these tests determine intrinsic mutagenicity of the metabolite. Table 1.3 compares results obtained with some of these metabolites. It is noteworthy that among arene oxides 4,5- and 9,10-epoxide show more activity than 7,8-epoxide. All 4 dihydrodiols 4,5-, 7,8-, 9,10- and 11,12- are weak mutagens. However all these metabolites need further metabolism to show tumorigenicity while diolepoxide acts as ultimate carcinogen. When dihydrodiols were tested, it was seen that 9,10-diol-7,8-epoxide was inactive while 7,8-diol-9,10-epoxide (BPDE) was active. However, mutagenicity of BPDE II was more than I in

TABLE 1.1

Skin tumor-initiating activity of BP derivatives in Charles River CD-1 mice

Initiator	Mice with tumors (%)	Tumors per mouse	Relative activity
BP	90 - 97	5.3 7.4-7.5	100
4,5-Oxide	24	0.24	5
7,8-Oxide	89	2.5	48
9,10-Oxide	15	0.15	3
11,12-Oxide	38	0.45	8
4,5-Dihydrodiol	30	0.30	4
7,8-Dihydrodiol	94	6.5	88
9,10-Dihydrodiol	13	0.10	1
2-Hydroxy	85	6.0	80
11-Hydroxy	80	2.1	28
1-,3-,4-,5-,6-,7-,8-, 9-,10-,12-Hydroxy	3 - 30	0.03-0.33	0.3 - 4
1,6-,3,6-,6,12-Quinones	21 - 29	0.20-0.30	3 - 4
7,8-Diol 9,10-epoxide II	7	0.07	1
7,8-Diol 9,10-epoxide I	69	1.5	28
6-Methyl	74	1.6	22
6-Hydroxymethyl	57	1.0	14

Thakker et al. (1985).

TABLE 1.2

Tumorigenic activity of BP derivatives in newborn mice

Compound	Dose (nmol)	Mice with pulmonary adenomas (%)	Number of adenomas per mouse
Experiment 1			
Control	-	1.2	0.13
BP	28	19	0.24
7,8-Dihydrodiol	28	66	1.77
7,8-Diol 9,10-epoxide II	28	10	0.14
7,8-Diol 9,10-epoxide I	28	86	4.42
Experiment 2			
Control	-	8	0.08
BP	1400	93	10.0
4,5-Oxide	1400	10	0.10
7,8-Oxide	1400	72	2.08
9,10-Oxide	1400	0	0
11,12-Oxide	1400	20	0.32
Experiment 3			
Control	-	8	0.08
BP	1400	81	6.40
2-Hydroxy-BP	1400	98	24.0
6-Hydroxy-BP	1400	11	0.11

Thakker et al. (1985).

microbial test while the reverse was observed in mammalian test.

In other tests, BPDE was shown to induce base insertion and deletion mutation in E.coli plasmid (Mizusawa et al. 1981). BPDE I and II were mutagenic in Salmonella test and in diploid human fibroblast cells but mutagenic efficiency of II was better than I in microbial cells and reverse was observed in human cells (Stevens et al. 1985). In general, in bacterial mutagenicity test, many carcinogenic and non-carcinogenic metabolites show positive response while limited studies with eukaryotic cells show apparent resemblance to carcinogenic picture.

BeP was mutagenic in bacterial test in presence of metabolizing enzymes. BeP showed positive response in few other tests viz. sister chromatid exchange and unscheduled DNA synthesis. In many other tests BeP was nonmutagenic (IARC 1983).

1.6. INTERACTION OF BP WITH CELLULAR MACROMOLECULES:

BP, once inside the body through any route of administration reaches cells of different organs and interacts with macromolecules of the cell viz. DNA, RNA, protein and membrane. Stowers and Anderson (1984) observed BP adducts with DNA and protein of various organs of mouse (oral) and rabbit (i.v.) e.g. liver, lung, forestomach,

brain, kidney, colon and muscle. Topical and oral administration of BP to mouse yielded BP-adducts with DNA, RNA and protein of lung,liver, skin and stomach (Morse and Carlson 1985). In rat i.p. administration of BP resulted in adducts with macromolecules of liver, lung, kidney (Prodi et al. 1970; Reeve and Gallagher 1981) and also of spleen and skin (Prodi et al. 1970). Even when cells in culture (Kuroki and Heidelberger 1971; Spelsberg et al. 1977; MacLeod et al.1979; Zytkovicz et al. 1981) or isolated nuclei (Vaught and Bresnick 1976; Pezzuto et al. 1976) were incubated with BP, adduct formation was observed with various macromolecules.

The nature of interaction between the carcinogens and macromolecules has been a much studied subject. From simple intercalation and ionic interactions to radical interaction, covalent linkages and cross-linkages have been suggested. However, Millers were the first to generalize that ultimate carcinogenic forms of most (if not all) chemical carcinogens are strong electron-deficient or electrophilic reactants (Miller 1970; Miller and Miller 1977). The electrophilic moiety of BP as depicted in Fig. 1.4 with carbonium ion (also called carbocation) at C_{10} position is highly reactive and will interact with any nucleophilic target site in the cells. (Fig. 1.5). The nature of the target may decide the criticality of interaction.

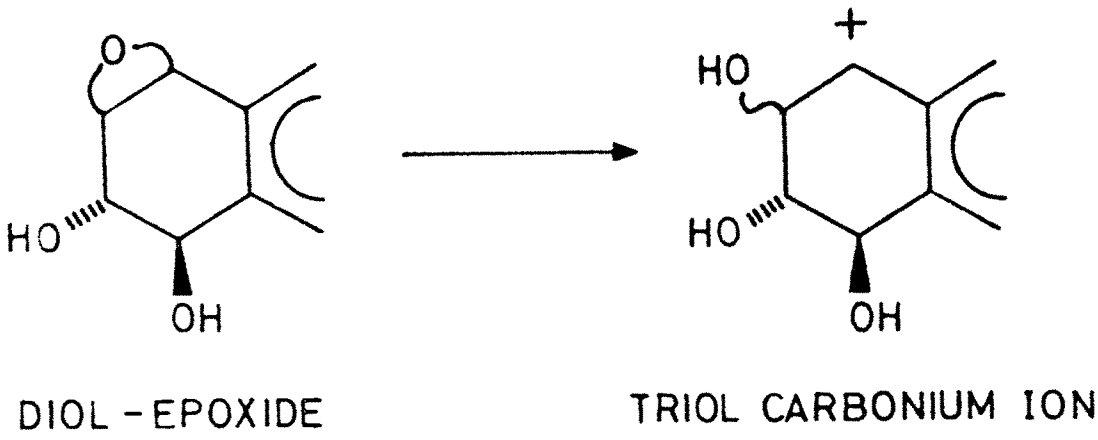


FIG. 1.4. Formation of carbonium ion from diol-epoxide. (Singer and Grunberger 1983).

CELLULAR NUCLEOPHILE $(a^{+}\text{--------}b^{-})$ + ELECTROPHILIC REACTANT $(X^{+}\text{-----}y^{-})$ ——— bx

DISPLACED ELECTROPHILE	NUCLEOPHILE	ELECTROPHILIC ATOM	LEAVING NUCLEOPHILE
NONE OR H^{+}	$>S$ MET	$-C^{+}<$	$R\text{-}SO_3^-$, $R\text{-}CO_2^-$, HSO_4^-
	$-SH$ CYS		$H_2PO_4^-$, Cl^-
	$>N-$ HIS(N-1, N-3)		$>N^-$, $-O^-$ from strained rings
	$>NH$ G(N-3, N-7, N^2) A(N-1, N-3, N-7) C(N-3)		
	$>CH$ TYR(C-3) G(C-8)	$>N^+$	$R\text{-}CO_2^-$, HSO_4^-
	$>C\text{-}OH$ TYR(O^4) G(O^6)		
	$>P\text{-}OH$ DNA		

FIG. 1.5. Nucleophilic sites in informational macromolecules attacked in vivo by electrophilic metabolites of various chemical carcinogens.
(Miller and Miller 1977).

The probable consequences of binding of BP to each of these macromolecules may be as follows. Binding to nucleic acids, in particular DNA, and its implications have been the subject of several reviews (Irving 1973; Sarma et al. 1975; Grunberger and Weinstein 1979a) and of books (Grover 1979; Singer and Grunberger 1983). This interaction may lead to toxicity, mutation, altered gene expression, carcinogenicity and other effects which will be discussed later. BP-binding to RNA could lead to alteration in gene expression (if bound to chromatin RNA), splicing and other post-transcriptional modifications (if bound to splice junctions of precursor RNA or to snRNA) and protein synthesis (if bound to tRNA, mRNA or rRNA).

Binding of BP to variety of proteins has been shown since the first demonstration by Miller (1951). Binding of BP to chromatin proteins like histones (Jenson et al. 1982; Kootstra 1982b; MacLeod et al. 1983) and nonhistone chromosomal proteins-NHCP (Kurokawa et al. 1982) or other nuclear proteins like tubulin (Prodi et al. 1970; MacLeod et al. 1982) has been observed. Binding of BPDE in vitro to histones and NHCP has been observed (Dock et al. 1986). Binding to cytosolic proteins by BP (Prodi et al. 1970; Soderkvist et al. 1986) may facilitate microsomal metabolism of BP (Hanson-Painton et al. 1981) or its delivery to target sites like estrogen-receptor (Ebright et al. 1986) or nuclear DNA

(Holder et al. 1981). Binding by BP to microsomal proteins (Thompson et al. 1986) and membrane-bound proteins (Wade et al. 1978) has been demonstrated. Specific interactions were shown with microsomal metabolizing enzymes like glutathione-S-transferase (Singh et al. 1985), a component of h-protein, and ligandin (Sarrif et al. 1976) and P-450 (Schelin et al. 1984; Marcus et al. 1985). BP is reported to interact with blood (cell) proteins (Nemoto 1986) and specific interaction has been shown with serum albumin (Roche et al. 1985) and hemoglobin (Shugart and Matsunami 1985). Binding of BP to nuclear enzymes like DNA polymerase, RNA polymerase, modifying enzymes and repair enzymes could bring about enormous consequences for the cell.

Binding of BP to cellular membrane (Brunette and Katz 1975), nuclear membrane (Ueyama et al. 1981) and model membranes (Lakowicz and Holden 1978) could alter availability, metabolism and transport of BP within the body and inside the cell.

1.7. ADDUCT FORMATION BETWEEN BP AND DNA:

1.7.1. General:

BP undergoes metabolic activation before binding to various macromolecules of the cell. In this, interaction of BP with DNA is most extensively studied. Brookes and Lawley

(1964) correlated carcinogenic potency of six hydrocarbons with their ability to bind to DNA or RNA but not to proteins. Since then, numerous studies as indicated in Table 1.4 have shown adduct formation in vivo between BP and DNA in various organs (skin, liver, lung, stomach and kidney) of animals. This adduct was also observed in other organs like spleen, colon and mammary glands of rats (Phillips et al. 1985) and brain, colon and muscle of mouse and rabbit (Stowers and Anderson 1985). This adduct was observed even when cells obtained from various organs of animals were grown in presence of BP (Table 1.5).

Efforts were made to simulate this in vivo adduct formation by carrying out microsome-mediated adduct formation in vitro between BP and DNA (Grover and Sims 1968; Gelboin 1969). At the same time Tso's group made attempts to link BP and DNA by chemical means using I_2 or H_2O_2 and Fe^{2+} (Lesko et al. 1969; Hoffman et al. 1970). However, the pattern of adducts with DNA, formed in microsome-mediated in vitro system was found to be identical to that observed in vivo with DNA of cells exposed to BP (Alexandrov et al. 1976; Jeffrey et al. 1977). Such similarity of adduct formation was observed when activated metabolites of BP such as BP-7,8-dihydrodiol and BPDE were reacted with DNA or synthetic polynucleotides (King et al. 1976b; Koreeda et al. 1976; Weinstein et al. 1976; Osborne et al. 1976a). Thus, the microsome-

TABLE 1.4

BP-DNA adduct formation in vivo

Organ	Animal	Route	References
1) Skin	Mouse	Skin	Brookes and Lawley 1964; Baer-Dubowska and Alexandrov 1981; Shugart et al. 1983; Morse and Carlson 1985; Huckle et al. 1986.
		Oral	Morse and Carlson 1985.
	Rat	Skin	Baer-Dubowska and Alexandrov 1981.
		i.p.	Prodi et al. 1970.
2) Liver	Mouse	Skin	Morse and Carlson 1985.
		Oral	Morse and Carlson 1985; Stowers and Anderson 1985.
		i.p.	Eastman et al. 1978.
	Rat	i.p.	Prodi et al. 1970; Viviani and Lutz 1978; Lutz et al. 1978; Reeve and Gallagher 1981; Phillips et al. 1985.
		i.v.	Boroujerdi et al. 1981.
	Rabbit	i.v.	Stowers and Anderson 1985.
	Fish	Oral	Varanasi et al. 1983.
3) Lung	Mouse	Skin	Morse and Carlson 1985.
		Oral	Morse and Carlson 1985; Stowers and Anderson 1985.
		i.p.	Eastman et al. 1978.
	Rat	i.p.	Prodi et al. 1970; Reeve and Gallagher 1981; Phillips et al. 1985.
		i.v.	Boroujerdi et al. 1981.
		Perfusion	Cohen et al. 1977; Vahakangas et al. 1979.
	Rabbit	i.v.	Stowers and Anderson 1985.

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TABLE 1.4 (contd.)

Organ	Animal	Route	References
4) Stomach	Mouse	Skin	Morse and Carlson 1985.
		Oral	Morse and Carlson 1985; Stowers and Anderson 1985.
	Rabbit	i.v.	Stowers and Anderson 1985
5) Kidney	Mouse	i.p.	Eastman et al. 1978
		Oral	Stowers and Anderson 1985.
	Rat	i.p.	Prodi et al. 1970; Reeve and Gallagher 1981; Phillips et al. 1985.
	Rabbit	i.v.	Stowers and Anderson 1985.

TABLE 1.5

BP-DNA adduct formation in cell cultures

Organ	Animal	References
1) Skin	Mouse	Shugart et al. 1983; Huckle et al. 1986.
2) Liver	Mouse	Vaught and Bresnick 1976.
	Rat	Burke et al. 1977; Jernstrom et al. 1978.
3) Mammary gland	Human	Stampfer et al. 1981; Pruess-Schwartz et al. 1986.
4) Colon	Human	Autrup et al. 1978.
5) Oesophagus	Human	Harris et al. 1979.
6) Embryo	Mouse	Baird et al. 1975; Brookes et al. 1975; Spelsberg et al. 1977; Shinohara and Cerutti 1977; Zytkovicz et al. 1981; Sebti et al. 1985.
	Hamster	Huberman and Sachs 1977; Ivanovic et al. 1978; MacLeod et al. 1979; Sebti et al. 1985.
	Rat	Sebti et al. 1985; Pruess-Schwartz and Baird 1986.
7) Lung or Trachea	Human	Harris et al. 1976; Yang et al. 1977; Feldman et al. 1978;
	Rat	Cohen et al. 1977.
8) Prostate	Mouse	Kuroki and Heidelberger 1971.
9) Endometrium	Human Mouse Rat	Kulkarni et al. 1986
10) BHK/Kidney	Baby hamster	Osborne et al. 1976b; Huberman and Sachs 1977.

mediated binding assay helped in establishing not only the in vivo route of adduct formation but also in tracing the metabolic activation route of BP. This in vitro system was also used in establishing metabolism and DNA binding in various organs using microsomes from skin of mouse (Buty et al. 1976), brain of rat (Das et al. 1985) and human placenta (Grilli et al. 1975). Liver microsomes from rat (Grover and Sims 1968; Grilli et al. 1975; King et al. 1975; Thompson et al. 1976; Jernstrom et al. 1976), hamster (Wang et al. 1972; Borgen et al. 1974) and fish (Ahokas et al. 1979; Nishimoto and Varanasi 1985) also facilitated the adduct formation. The in vitro system was used in establishing the mechanism of interaction between BP and DNA by using a variety of substrates like chromatin, and synthetic polynucleotides apart from purified DNA.

Ability of other subcellular particles to facilitate the adduct formation in vitro between BP and DNA has been demonstrated for isolated nuclei (Rogan and Cavalieri 1974; Alexandrov et al. 1976; Jernstrom et al. 1976; Pezzuto et al. 1976; Rogan et al. 1976; Baer-Dubowska and Alexandrov 1985) and mitochondria (Niranjan et al. 1985). This proves the existence of metabolizing system in these subcellular organelles independent of microsomal systems and explains increased chances of macromolecules from these subcellular fractions interacting with metabolites of BP.

1.7.2. Detection and characterization of the BP-DNA adduct:

The detection, quantification and characterization of BP-DNA adduct formed either in vivo or in vitro has been carried out in a variety of ways. The most obviously used method for detection is through the use of radioactive carcinogens as described by Baird (1979). The adduct formed can be detected in isolated DNA or even in individual nucleotides or nucleosides obtained after enzymatic hydrolysis and separation by Sephadex LH-20 or HPLC (Baird 1979). These studies also indicated nature of the adduct to be much more than intercalation. Since presence of a bulky chromophore like BP would change the conformation of DNA, such changes have been studied by physical techniques like UV spectrum, electric linear dichroism (Singer and Grunberger 1983), CD spectra (Chen 1985) and magnetic resonance (Chiha et al. 1975; Lefkowitz et al. 1979). The fluorescing property of BP has been employed very effectively to detect and characterize the adduct (Vigny and Duquesne 1979). Sensitivity of this technique has been increased by many variations viz. low temperature (-70^{o}) fluorescence (Ivanovic et al. 1976), electrofluorescence (Ridler et al. 1986) and synchronous fluorescence (Vahakangas et al. 1985).

Post-labeling is another method to detect modified nucleotides (Singer and Grunberger 1983). Unusual ability of carcinogen-modified DNA to bind to cell is a detection method developed by Kubinsky et al. (1981). Raising of

antibodies against BP or BPDE-modified DNA or specific regions of DNA has opened up a very sensitive field of immunoassay of the adduct. It has been used cytologically to visualize modified regions of the genome (Slor et al. 1981) or to differentiate adduct with single or double stranded DNA (Muller and Rajewski 1981). The immunological quantification of the adduct has been attempted with radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). A more sensitive combination, ultrasensitive enzymatic radioimmunoassay (USERIA), was shown to detect upto 1 adduct per 7×10^6 nucleotides (Singer and Grunberger 1983). Use of specific monoclonal antibodies in these techniques has helped in fine tuning the detection of specific adducts of isomers of BPDE with DNA, RNA or synthetic polynucleotides (Santella et al. 1986).

1.7.3. Mechanism of interaction of BP with DNA:

In order to understand the biological effects like toxicity, mutagenicity and carcinogenicity of BP and its metabolites, it was a must todecipher the interaction of these compounds with DNA. The knowledge of various aspects of BP-metabolism and the known toxic, mutagenic and carcinogenic effects of these metabolites (discussed earlier) helped in arriving at conclusion regarding the major biologically relevant or important route of metabolic activation of BP and binding to DNA. Existing knowledge regarding these has

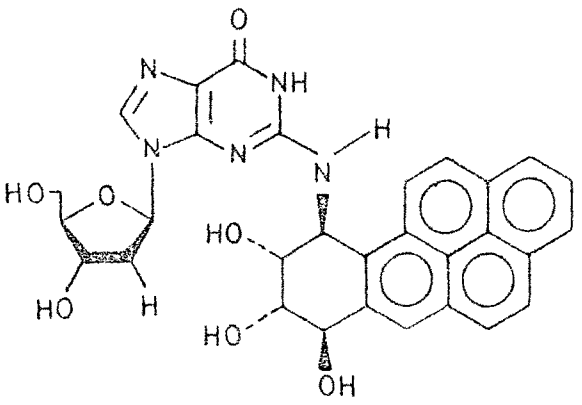
been summarized (Phillips and Sims 1979; Sims and Grunberger 1983; and Thakker et al. 1985) as follows. Proximate carcinogen BP-7,8-dihydrodiol binds (to DNA) better than BP. Both the diastereomers of the ultimate carcinogen BPDE react with nucleic acids and that C_{10} is the position of BP at which major binding occurs. BPDE I and II, both are added in trans and cis configurations. However, there appears to be time-dependent existence of the adduct in cell cultures (Thakker et al. 1985) and in epidermis (Pelling and Slaga 1982), i.e. early adduct formation with BPDE-II (4-6 h) and later with BPDE I.

At the same time, noncovalent interaction, notably intercalation was being consistently observed by many workers (Meehan et al. 1982; Geacintov et al. 1982; MacLeod and Zachary 1985). Another confusing observation was that DNA acts as catalyst in hydrolysis of BPDE to tetraols which also intercalate with DNA (Harvey 1981). Geacintov (1985) offered with spectral evidence an explanation that BPDE adducts with DNA are of 2 types: Site I adduct which is intercalative type and site II adduct which is covalent adduct with orientation of planar aromatic residues tilted closer to the axis of the helix. Both BPDE I and II form site I adduct which undergoes specific and general acid catalysis to form tetraols (90%) and covalent adducts (10%). Moreover he also showed that (+)optical enantiomer of BPDE I undergoes marked reorientation after covalent binding to form almost exclusively site II

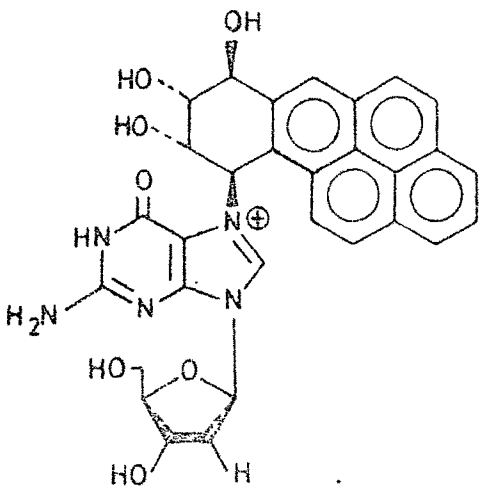
adducts, while (-)BPDE I and (+) and (-)BPDE II gave mixture of both site I and site II adducts. These might help in explaining the in vivo observations from several studies that major adduct observed is with BPDE I and that too with (+)enantiomer of BPDE I (Thakker et al. 1985).

Nonetheless, the interpretation that interaction of carbocation at C_{10} arising from (+)BPDE I with DNA, gives rise to the biological effects of BP has to be stated with due caution, as there has been persistent but infrequent reports on importance of other positions of BP in DNA binding and other biological effects. BP-binding has been shown with 9-hydroxy BP (King et al. 1976a), BP-semiquinone radical (Kodama et al. 1977) and 6-oxy BP radical (Kodama and Nagata 1977). Cavalieri and Rogan (1985) have observed in vivo binding of C_6 position of BP to guanine in mouse skin DNA, emphasizing the role of radical cations of BP in DNA-binding and carcinogenesis.

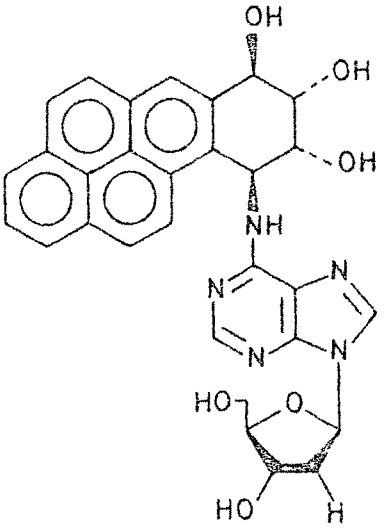
The site on DNA at which the adduct formation with BP occurs is another area in which many observations and interpretations are available. Sims and Grunberger (1983) and Thakker et al. (1985) summarized the dominant view that major adduct was observed at N^2 of G (Fig. 1.6) in which G residue is trans to 9-OH and the 8 and 7-OH are also trans. Since N^2 is relatively exposed in the minor groove of the helix, binding of BPDE with N^2 occurs in the minor groove



dG-N^2-BPDE I



dG-N-7-BPDE I



dA-N^6-BPDE I

FIG. 1.6. Adduct formation of BPDE with DNA. (Singer and Grunberger 1983).

with little distortion of native DNA conformation (Grunberger and Weinstein 1979b). There has been general observations consistent with this interpretation. Mengle et al. (1978) observed binding of BPDE in the GC rich region of phage DNA. The major adduct of BP-modified DNA was with G (Meehan et al. 1977; Kakefuda and Yamamoto 1978). Using (+) and (-)isomer of BPDE I with single (SS) and double-stranded (DS) DNA, Meehan and Straub (1979) observed that (+)BPDE I bound more than (-)isomer to G of DS DNA but no such difference was observed with SS DNA,

There have been observations regarding aduct formation with positions of DNA other than N^2 of G. Yang et al. (1983) showed base specific intercalation of BP-7,8-dihydrodiol not with poly (dG-dC) but with poly(dA-dT) leading to strand breaks. Adduct formation of N-7 of G (Fig. 1.6) has also been observed (Osborne et al. 1978; King et al. 1979), but it renders glycosidic bond of nucleoside so labile that depurination occurs, making it difficult to isolate this adduct (Osborne and Merrifield 1985). Adducts were also observed with N^6 of A as shown in Fig. 1.6 (Jeffrey et al. 1979) and with C (Meehan et al. 1977). N^6 of A, however, lies in major groove of DNA helix and hence adduct formation at this amino group may lead to different set of conformational changes in DNA as compared to adducts at N^2 of G (Grunberger and Weinstein 1979b). Binding by BP to

phosphodiester backbone as suggested by Gamper et al.(1977) and Koreeda et al. (1976) leads to strand break.

These variety of interactions may have a role to play in determining the susceptibility and specificity of different organs of animals to carcinogenesis, mutagenesis and toxicity by BP.

1.8. BIOCHEMICAL CONSEQUENCES OF BP-ADDUCTS WITH NUCLEIC ACIDS

Three major biological effects which were observed after treatment with BP viz., toxicity, mutagenicity and carcinogenicity, could be explained as a result of adduct formation by BP with macromolecules, especially DNA (McCormick and Maher 1985; Swenberg et al. 1985). Adduct formation with DNA could lead to local denaturation, unwinding, depurination, SS and DS breaks. These alterations could lead to modifications in biochemical functions of the cell viz. DNA, RNA and protein synthesis. However, the earliest response of the cell to damage of its DNA by chemical carcinogens is activation of repair systems i.e. DNA-excision repair and associated unscheduled DNA synthesis. Sensitivity of X. pigmentosum cells to mutagenesis and cell killing by PAH highlights importance of repair mechanism in PAH carcinogenesis (Grunberger and Weinstein 1979a). Repair of damaged DNA has been observed in vivo (DiGiovanni et al. 1985) and in cell

culture (Cerutti et al. 1978; Kaneko and Cerutti 1982; Kootstra 1982a) as removal of adducts. Stowers and Anderson (1985) suggested differential removal (or persistence) of adducts in different organs of the body as one of the factors determining susceptibility towards the carcinogen.

DNA synthesis in response to BP-treatment was found to be altered in all directions i.e. from inhibition in perfused lung of pretreated rats (Imamura et al. 1982) and reversible early inhibition in cells (Bowden et al. 1982; Novicki et al. 1985) to stimulation in rat hepatocytes after BP-treatment for 1-2 days (Stairs et al. 1983). The in vitro DNA synthesis was shown to be inhibited on binding to DNA by BP (Yamaura et al. 1978) or BPDE I (Mizusawa and Kakefuda 1979). This was shown to be due to premature chain termination during DNA synthesis (Yamaura et al. 1981). Phage DNA replication in host cell was reversibly inhibited by BP-7,8-dihydrodiol (Chang et al. 1979) and BPDE (Chang et al. 1979; Rinaldy et al. 1982). Transfectional activity of DNA and RNA phages was reduced by BPDE (Grunberger and Weinstein 1979a). Mitochondrial DNA replication was also reduced by BP (Stairs et al. 1983) and this was shown to be due to effect of BP on template and not on the enzyme (Salazar et al. 1982).

Effects of BP-binding to DNA, chromatin and to components of transcription machinery on the process of transcription will be discussed in Chapter II.

Effects of BP on protein synthesis could be due to its binding with RNA (mRNA, tRNA or rRNA) or with proteins (enzymes, ribosomal and other proteins). Initiation of translation for globin mRNA and translation efficiency of phage RNA were reduced by BPDE (Grunberger and Weinstein 1979a). Modification of tRNA and rRNA was observed with BP-metabolites (Pietropaolo and Weinstein 1975) and modified initiator tRNA showed stimulated aminoacylation (Hradec and Kolar 1984).