GENERAL INTRODUCTION

Cancer is a disease of cells occurring in multicellular organisms. The cancerous cells display such unique behavioural traits as uncontrolled proliferations, invasion and metastasis. More than hundred forms of cancers have been found in tissues or organs of the body and in the blood and lymphatic system. Table 1 shows the rank order of incidence of some of the most common cancers among males and females all over the world.

CAUSES OF CANCER AND MECHANISM OF CARCINOGENESIS

The process of carcinogenesis is understood to be multifactorial, multistep and multigenic. Several extrinsic as well as intrinsic factors have been attributed for the incidence of cancer. While the intrinsic factors involve the genetic factors and hormonal interference, the external factors involve the exposure to different carcinogens which mainly include chemicals, radiation and viruses.

All these factors exert their effect by damaging DNA in one way or other. The accumulation of genetic damage at the somatic level is found to be responsible for the cancerous changes. The recent increased understanding of
oncogenes and antioncogenes provides the molecular basis for carcinogenic processes.

**Radiation and Cancer**

The most insidious physical cause of cancer is radiation. Friben (1902) first suggested that radiation is carcinogenic. Ionizing radiations are known to produce somatic mutations, activate oncogenic viruses or alter protooncogenes and cause misrepairs in nucleic acid sequences which results in neoplastic changes (Hiatt et al., 1977). Mehr and McCormick (1979) reported the induction of pyrimidine dimers in DNA by ultraviolet rays. The expression of various oncogenes eg., myc, k-ras, N-ras, H-ras, and abl have been detected in radiation induced tumors or transformed cells (Borek, 1985; Kaminsky et al., 1985; Sarvey and Garte, 1986; Mizuki et al., 1985). The mechanism of radiation - induced oncogene activation is however not exactly known.

**Viruses and Cancer**

Transformation of cultured cells by polyomavirus (Vogt and Dulbecco, 1960) led to the investigation of virus-induced cell transformation at the molecular level. The unique ability of oncogenic viruses to transform cells,
using the encoded information in one or few transforming genes, distinguishes them from other viruses. There are some evidences linking specific viruses within the complex pathway of the development of Burkitts' lymphoma in Africa and nasopharyngeal carcinoma in Far East (Epstein-Barr Virus). Human papilloma viruses (HPV) have been associated with the occurrence of human genital cancer (zur Hausen, 1976). HPV types, 6, 11, 16 and 18 have been implicated in the development of cervical dysplasia and invasive carcinoma of the cervix, although their exact role is not fully known.

The oncogenic viruses may contain either DNA or RNA. DNA of the DNA virus, after entering the cell, gets integrated into the genome of the host cell and this is followed by the change to cancer cell. The RNA of the RNA tumor viruses acts as a template for the RNA dependent DNA polymerase that is present in the virion. The DNA product is then incorporated into the cellular DNA as with oncogenic DNA viruses and replicates with the host DNA.

Recent investigations in the field of viral oncology are focussed on the functions of transforming proteins encoded by the viral oncogenes and these have shed light in understanding the mechanism of transformation at the cellular and molecular levels.
Chemicals and Cancer

The carcinogenic effect of chemical compounds was suggested as early as 1775, when Percival Pott noticed that boys who swept chimneys had a very high incidence of squamous cancer in the skin around scrotum. High incidences of bladder, lung and liver cancers have been observed among the workers of chemical industries. The workers in the aniline dye industries are susceptible to develop cancer of the urinary bladder. Environmental chemicals have been held responsible for the incidence of about 70% of known cancers (Epstein 1977).

In 1915 Yamagawa and Ichikawa reported the first production of skin tumors in animals by the application of coal tar to the skin. These tumors were malignant in nature and gave the concept of experimental chemical carcinogenesis.

Depending upon the reactivity of the parent compound with cellular nucleophiles, the carcinogens are classified as direct acting and indirect acting. The majority of chemical carcinogens, other than the direct-acting ones, require metabolic activation by different metabolizing enzymes in the host to yield reactive intermediates known as ultimate carcinogens, without which they are noncarcinogenic.
and nonmutagenic. The ultimate carcinogens are highly reactive electrophiles that react with nucleophilic macromolecules in the cell, the critical target most probably being DNA (Miller, 1978). The direct acting or activation-independent carcinogens possess the necessary chemical structure for their reactivity as electrophillic reactants. Some of the example of direct acting carcinogens are \( \beta \)-propiolactone, propane sulfone, some nitrogen mustard derivatives, alkyl and other sulfate esters and some active halogen derivatives such as bis (Chloromethyl) ether (Van Duuren et al., 1979; Weisburger and Williams, 1980; Zajdela et al., 1980).

On the basis of the mechanism of action the carcinogen can again be either genotoxic, which interacts covalently with the genetic material of the cell especially DNA, or epigenetic which does not react with DNA (Table 2). The genotoxic carcinogen results in an alteration in primary DNA sequence example a single base pair change, a deletion, an insertion, a rearrangement or duplication of one or more base pairs while epigenetic carcinogen do not cause any alteration in DNA sequence and brings about alteration in DNA methylation, transcriptional activation, translational control etc.
Table 1: Rank Order of Incidence of Ten Most Common Cancers in World

<table>
<thead>
<tr>
<th>Rank</th>
<th>Males</th>
<th>Females</th>
<th>Both sexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lung</td>
<td>Breast</td>
<td>Stomach</td>
</tr>
<tr>
<td>2</td>
<td>Stomach</td>
<td>Cervix uteri</td>
<td>Lung</td>
</tr>
<tr>
<td>3</td>
<td>Mouth/pharynx</td>
<td>Stomach</td>
<td>Breast</td>
</tr>
<tr>
<td>4</td>
<td>Colon/rectum</td>
<td>Colon/rectum</td>
<td>Colon/rectum</td>
</tr>
<tr>
<td>5</td>
<td>Esophagus</td>
<td>Lung</td>
<td>Cervix</td>
</tr>
<tr>
<td>6</td>
<td>Prostate</td>
<td>Mouth/Pharynx</td>
<td>Mouth/Pharynx</td>
</tr>
<tr>
<td>7</td>
<td>Liver</td>
<td>Esophagus</td>
<td>Esophagus</td>
</tr>
<tr>
<td>8</td>
<td>Bladder</td>
<td>Lymphatic</td>
<td>Liver</td>
</tr>
<tr>
<td>9</td>
<td>Lymphatic</td>
<td>Liver</td>
<td>Lymphatic</td>
</tr>
<tr>
<td>10</td>
<td>Leukemia</td>
<td>Leukemia</td>
<td>Prostate</td>
</tr>
</tbody>
</table>

From Pitot (1989).

Table 2: Classification of Carcinogenic Chemicals

<table>
<thead>
<tr>
<th>Carcinogen Class</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotoxic</td>
<td></td>
</tr>
<tr>
<td>Activation-independent</td>
<td>Alkylating agent</td>
</tr>
<tr>
<td>Activation-dependent</td>
<td>Aromatic Amines</td>
</tr>
<tr>
<td></td>
<td>PAH</td>
</tr>
<tr>
<td></td>
<td>Metal</td>
</tr>
<tr>
<td>Inorganic*</td>
<td></td>
</tr>
<tr>
<td>Epigenetic</td>
<td></td>
</tr>
<tr>
<td>Solid state</td>
<td>Plastic, asbestos</td>
</tr>
<tr>
<td>Hormone</td>
<td>Estrogen, Androgen</td>
</tr>
<tr>
<td>Immunosuppressor</td>
<td>Purine analog, antibody</td>
</tr>
<tr>
<td>Cocarcinogen</td>
<td>Phorbol ester, Catechol.</td>
</tr>
<tr>
<td>Promoter</td>
<td>Phorbol ester, Phenobarbital</td>
</tr>
</tbody>
</table>

* Tentatively categorized as genotoxic because of some evidence for direct interaction of some members with DNA. From Becker (1982).
The complex process of carcinogenesis has been operationally divided into three stages: initiation, promotion and progression. The process of chemical carcinogenesis can also be segregated into pharmacokinetic and pharmacodynamic phases. The sequential molecular and cellular events involved in the whole process of chemical carcinogenesis is believed to occur as a result of interplay of genetic and epigenetic changes or aberration (Fig. 1).

The whole process of chemical carcinogenesis can be summarized as follows (Fig. 2):

a) Interaction of an ultimate carcinogen with the specific site of cellular macromolecules especially DNA, leading to its damage. This lesion can be repaired by repair enzyme systems and the normal condition is restored (Fig. 3).

b) Replication of the altered DNA leads to the fixation of carcinogenic damage by causing mutations. Mutations in cellular DNA affecting certain genes, protooncogenes and antioncogenes normally involved in the control of cell growth, permit a normal cell to become cancerous (Ricketts 1990).

c) Clonal expansion of the initiated cells is accomplished by promoters which increases the carcinogen response.
Fig. 1. Mechanism of action of chemical carcinogens. Genotoxic carcinogens induce mutations and epigenetic carcinogens modulate the expression of DNA lesions (From Lutz and Mair, 1988).
PROCARCINOGEN → DETOXIFICATION → EXCRETION

BIOACTIVATION

ACTIVE ELECTROPHILES

INTERACTION WITH DNA, RNA, PROTEIN

ALTERED MACROMOLECULE

REPAIR

NORMAL CELL

REPLICATION

FIXED CARCINOGENIC DAMAGE (MUTATION)

INITIATED CELLS

GROWTH PROMOTION

TRANSFORMED CELLS

PROGRESSION

CANCER CELL

Fig. 2 - Important Steps in Chemical Carcinogenesis
Fig. 3  Carcinogen damage to DNA and Repair
d) The final step in the development of cancer is the progression of transformed cells (Williams and Weisburger, 1986) to cancer cells. Progression is characterized by demonstrable changes in the neoplastic cells associated with increased growth rate, increased invasiveness, metastasis and biochemical and structural characteristics of the neoplasm. These alterations correlate with changes in the number or arrangement of genes or with visible chromosomal alterations within a majority of the neoplastic cells that make up the tumor.

HORMONES AND CANCER

Another very important class of carcinogens includes exogenous or endogenous hormones in the animals or human body. The very concept of hormones as a causative factor in the incidence of cancer comes from Beatson (1896) who suggested a relation between breast cancer and the ovary. Realization of the fact that about 25 percent of human tumors originate in endocrine tissues or their target organs and an unusual occurrence of vaginal cancer in young women exposed to diethylstilbestrol (DES) in utero (Herbst, et al., 1971) leads to the recent emphasis and extensive investigation of hormonal involvement in the initiation and
growth of different human cancers. One of the major concerns in relation to the use of steroids, as contraceptives or replacement therapy, has always been the possible stimulation of a preexisting malignancy or the development of a new one. Of the various hormonal agents, sex hormones, particularly estrogens have been the most implicated in cellular transformation. Estrogen has been shown to induce cancer of different organs in a numbers of animals (Li and Nandi 1986). Correlations have been made between oral contraceptives and other prescribed estrogens and endometrial, hepatic, cervical, ovarian and breast cancer. (Hoover, 1981 and Henderson, 1982). Epidemiological as well as experimental studies regarding the hormonal involvement in cancer incidence have resulted in a growing awareness of the carcinogenic potential of both natural and synthetic hormones.

Hormones have been found to play a very important role in maintaining the internal millieu of the body. Tumor induction in endocrine organs or their target tissues as a result of the exogenous hormones administration and endocrine gland ablation with or without other agents (such as oncogenic viruses, chemical carcinogen, ionizing radiation etc.) appears to be because of a severe disruption in normal homeostasis.
In addition to the direct involvement of hormones in tumor induction they may also act in concert with other carcinogenic agents. Berenblum (1947) suggested that hormone can have modifying or permissive influence on cancer. Bittner (1956) demonstrated three factors essential for the production of mammary carcinoma in mice - genetic susceptibility, hormonal influence and a virus transmitted through the milk.

Possible roles of hormones in Carcinogenesis

Considering that hormones may not be carcinogenic by themselves it is suggested that this might modify the host and/or target tissue after the initiation caused by different carcinogenic agents such as chemicals viruses or ionizing radiations. These modifications might involve:

a) Promotional and/or cocarcinogenic effects
b) Modification of the host immune system.
c) Activation of viruses.
d) Modification of receptors for carcinogens and/or metabolic conversion of a procarcinogen into an ultimate carcinogen in the target cells.
e) Regulation of tumor cells growth and/or initiation of DNA synthesis following carcinogenic stimulus.
Besides these, hormones may as well act as an initiator of carcinogenesis (Horning et al., 1978; Purdy, 1984; Li and Li, 1984; Metzler 1984 and Lacassagne, 1932) by a number of ways eg. by acting as a mutagen, by increasing the frequency of cell replication in target tissues, which in turn increases the probability of error in DNA copying, by inducing chromosomal abnormalities, and/or producing metabolites of the hormones which act on DNA or other macromolecules.

Riegel and Mueller (1954) demonstrated the formation of a protein-bound metabolite of estradiol. Jaggi and his associates (1978) have demonstrated a very low level covalent binding of synthetic estrogen to DNA. Estrogen has been found to be capable of undergoing orthohydroxylation to form catechols which are subsequently oxidizable to reactive species (Li et al., 1985). Although covalent binding of oxidative reactive estrogen metabolites to DNA and microsomal proteins have been demonstrated (Tsibris et al., 1977) but its relationship to mutagenicity or carcinogenicity is not clear.

Not much is known about the oncogene activation and expression in hormonal carcinogenesis. Prolonged hormonal stimulation might cause inappropriate expression of
autocrine growth factors or inappropriate activation of their receptors, thus allowing a normal cell to become neoplastic.

CERVICAL CANCER

Cervical cancer on a global basis is the second most common cancer among women. In 450 B.C. Hippocrates mentioned about the cancer of uterus in his writing where he described the diseases of women. The first observations relating to the incidence, distribution and possible causative factors of carcinoma of the uterine cervix were made in 1842 by Rigoni-Stern.

The regions of the world where the risk of getting cervical cancer is highest are Sub-Saharan Africa, Central and South America and South East Asia, where cancer of the cervix constitutes 20-30% of all cancers in women. The highest recorded incidence rates occur in South America and particularly north-eastern Brazil, with age standardized rates of 83.2 in Recife and 46.5 in Fortaleza, and in Colombia (Cali) with a rate of 48.2 (IARC, 1990). Some developed countries like New Zealand (Maori population), East Germany, Romania, Singapore and Hong Kong also have considerably high rates of cervical cancer incidence.
Nigeria, Zimbabwe, Japan, Israel, Switzerland and non-Maori population of New Zealand show a very low cervical cancer incidence. Low incidence of this cancer is also reported among the Malaysian Orang Asli aborigines (Sumithran, 1976) and among Mormon Women in Utah (Gardner and Lyon, 1977). Within California the range in incidence between the Japanese (low) and the Spanish (high) is five fold.

In India cervical cancer incidence is very high and develops in about 90,000 women annually (WHO 1986). Among different Indian States, Madras shows the highest incidence of cervical cancer (Annual Report, National Cancer Registry Progress ICMR, India, 1987).

**Risk factors**: The etiological factors responsible for the development of cervical carcinoma have been the subject of considerable speculation. The results of a large number of epidemiological studies suggest several risk factors:

a) Early age at first intercourse  
b) HPV Infection  
c) Low socio-economic status  
d) Oral contraceptive pill use  
e) Multiple Sexual partners  
f) Parity  
g) Poor Hygiene.
Sexual behaviour is highly related to the risk of cervical neoplasia (Rotkin 1973, Harris et al. 1980, Singer 1982, 1983). Factors like early age at first intercourse, multiple partners, unstable marriage status, and early age of marriage have been well documented in the past. There are two behavioural variables that are now worth considering in details namely age at first intercourse and number of sexual partners. Early adolescence coitus has been found to be a period of heightened vulnerability to this disease (Rotkin, 1967). Multiple partners have been found to produce three fold increase in the risk of cervical cancer as compared to women with one partner (Terris, et al., 1967; Martin, 1967). Smegma has also been suggested to have some carcinogenic action and the presence of some chemical carcinogen in it has been proved experimentally (Alexander, 1973). The possible role of ritual circumcision is still unclear (Persuad, 1977) and the strikingly low incidence of cervical carcinoma in Jewish women might be due to other causes.

Perhaps among all cancers, cervical cancer is most linked with socioeconomic condition (Clemmesen, 1977 and Kennaway, 1948). The obvious explanation for this difference could be the difference in sexual behaviour and hygiene. In Brazil cervical cancer is much more frequent in northeastern regions than in southeast, a fact that correlates again with
low socio-economic status, poor hygiene and higher promiscuity in the Northeast (Gardner and Lyon, 1977, Lopes de Faria et al., 1982).

Parity as a risk factor has been argued about for many years and no definite consensus has been reached. However Miller et al., 1980, in a study of over 11,000 women in Canada showed that parity produced an increased risk for in situ carcinoma of the cervix at all ages but for invasive cancer it only operated in parous women under the age of 50.

Role of HPV infections in anogenital cancer had been postulated in 1976 (zur Hausen, 1976, 1977) and then the DNA of the first genital HPV (HPV6) was described in 1980 (Gissmann and zur Hausen) from a genital wart (Condyloma-acuminatum), cloned and characterized (de Villiers et al., 1981; Schwarz et al., 1983) and subsequently sequenced (Schwarz et al., 1983). Later in 1982 Gissman isolated a closely related DNA (HPV 11) from laryngeal papilloma and genital warts (Gissmann et al., 1982). Besides this DNA of HPV 16 and 18 have been directly cloned from cervical cancer biopsy. A large no. of evidences have been published in past few years suggesting an etiological role of Human papillomavirus (HPV) in cervical intraepithelial neoplasia and cervical cancer. These findings include the frequent coexistence of HPV and cervical intraepithelial neoplasia in
cervical biopsies and pap smears, malignant transformation of certain HPV lesions, as well as the association of HPV infection with cervical cancer as sexually transmitted diseases (Gissmann, 1984; Kadish et al., 1986; Knox et al., 1988; Koss 1987; Meisels et al., 1976; Mitchell et al., 1986; Munoz et al., 1988; Syrjanen, 1983, 1984, 1985).

Herpes simplex virus, which was once considered as an important factor for cervical cancer incidence, has been suggested possibly to interact with persisting HPV infection in two directions: by mutating specific host cell genes and destroying the intracellular surveillance for HPV transcription, or by amplifying persisting HPV genome (zur Hausen et al., 1984).

The possibility of synergistic effects have been suggested between chemical and physical carcinogens and papillomaviruses (zur Hausen 1977b) which can be explained by considering their possible action on Cellular Interfering Genes, or on viral protein binding domains, controlling HPV transcription.

Recently a possible association of Human immunodeficiency virus-induced immunosuppression and HPV infection in the genesis of cervical intraepithelial neoplasia has been reported (Michelle et al., 1989).
Smoking has also been suggested as a risk factor for cervical cancer incidence (Harris et al., 1980). A risk of some 13 times was found in women who had smoked (Buckley et al., 1981). Wigle et al., (1980) however found a 4 fold risk for development of cervical intraepithelial neoplasia. It has been suggested that nicotine by its epithelial toxic effect might facilitate the entry of possibly a viral mutagen (Singer 1985).

There are several other factors, associated with males, which are considered to be responsible for increased risk of developing cervical neoplasia in the female partners. In 1976 Singer, Reid and Coppleson referred these groups of individuals as high risk males. These factors include social class, penile cancer, basic protein on sperm surface and sexual behaviour. The basic proteins have a profound effect on the cell surface due to its ability to bind nuclear DNA.

Use of oral contraceptive steroids has been the subject of debate as to whether they increase the woman's risk of cervical cancer. Although the epidemiological studies have yielded equivocal results regarding the possible association between oral contraceptive pill use and cervical cancer a number of recent prospective studies have shown trends of increasing cervical cancer with extended duration of pill use (Andolsek, 1983; Beral, 1988; Vessey 1983; Brinton,
Beral (1988) reported that the incidence of cervical cancer after 10 years of pill use was more than 4 times than in nonusers. Despite these positive findings the relationship of oral contraceptives to risk of cervical cancer remains unresolved, with several studies concluding that a true causal effect is unlikely.

Some indirect role of O.C. pill has also been suggested in the cervical cancer incidence. Vessey (1986) is of opinion that O.C. pill hormones could make the cervical epithelium more susceptible to the sexually transmitting agents that can cause cervical cancer. Interaction of OC pill hormones with the hormone-responsive element in the non-coding region of genital HPVs could lead to increased virus production and probably also to enhanced proliferation of viral DNA carrying cells (zur Hausen, 1989).

Like oral contraceptive pills, injectable contraceptive steroids have also generated a lot of controversies. One of the major issues raised by the scientific community as well as social organizations has been the possible association of injectable contraceptive use and neoplasia. Medroxy progesterone acetate (MPA) in its microcrystalline form and Norethisterone enanthate (NE) are the two progestin preparations that have been used popularly in the form of
injection to provide an effective single dose contraception for 1-3 months.

Some of the epidemiological studies have suggested a possible increased risk of getting cervical cancer with the use of injectable contraceptives. A few animal studies have shown the occurrence of mammary and endometrial tumor following long term administration of medroxyprogesterone acetate (MPA) but we don't get any reason to associate MPA use with increased risk of neoplasia.

DRUG METABOLIZING ENZYMES AND BIOTRANSFORMATION

The biotransformation of xenobiotic compounds including carcinogens, is accomplished by a number of drug metabolizing enzyme - catalyzed reactions which are of two types: Phase I reactions, which add or expose functional groups (e.g. -OH, -SH, -NH₂, -COOH) in the xenobiotic compounds and convert them into more water soluble derivatives while phase II reactions involve the covalent binding of phase-I derived metabolites to an endogenous molecule, such as sulfate, producing a conjugate which can be easily excreted. Figure (4) shows phase I and phase II biotransformation reactions. Liver has been found to be the principal organ for all these enzyme systems.
Fig. 4 Integration of phase I and phase II biotransformation reactions (From Sipes and Gandolfi, 1986).
Unfortunately detoxication is not always the end result of biotransformation. In a number of cases the metabolic products or the intermediate products formed in the course of biotransformation are more toxic or carcinogenic than the parent compound. In case of a number of chemical carcinogens, it is the reactive intermediates which initiate the events in the process of carcinogenesis.

Besides phase I and II drug metabolizing enzymes, glutathione (GSH) is the most important non-protein thiol in the animal cell and plays a key role in drug biotransformation and prevention of drug toxicity. In mammalian cells GSH concentration is very high. Most of the intracellular GSH exist in thiol form and is localized in the cytosol.

Thus any substance with the potential of altering the phase I and II drug metabolizing enzymes and glutathione level can make the host more susceptible or resistant to different chemicals including carcinogens.

Phase I Enzymes

1. Cytochrome P450 System

The cytochrome P-450 (Cyt. P450) system is a group of enzymes (E.C.1.14.14.1) primarily responsible for the
biotransformation of xenobiotics. The P450s catalyze the oxidation of exogenous as well as endogenous lipophilic compounds to more polar metabolites which in turn facilitate their detoxification and subsequent elimination from the living system. These enzymes are also involved in the biosynthesis of steroids, bile acids and vitamins (Fig. 5). The reactions which they catalyze is mixed function oxidation:

\[ S + \text{NAD (P) H + O}_2 \rightarrow \text{SO + NAD (P)}^{+} + \text{H}_2\text{O} \]

where S is the substrate and SO is the oxidized product (Nebert, 1989; Guengerich, 1990).

The P450s are predominantly found in the endoplasmic reticulum, mitochondria and nuclear envelope of the liver, although they are also present in the brain, adrenal, kidney, gonads and skin (Sipes and Gandolfi, 1986).

In the endoplasmic reticulum, the Cyt. P-450 system is made-up of two enzymes.

1) A flavoprotein - NADPH Cyt. P450 reductase which is responsible for the transfer of electron(s) from NADPH to Cyt. P450. This enzyme has a molecular weight ranging from 74,000 to 80,000 depending on the animal species (Guengerich and Liebler, 1985).
Fig. 5 Functions of mammalian cytochrome P450 (From Wolf, 1986)
2) A heme containing protein - Cyt. P450 which functions as the terminal oxygenase of the enzyme system. The heme protein consists of an iron protoporphyrin IX heme moiety which is noncovalently bound to a single polypeptide chain (Murray and Reid, 1990). The molecular weight of this enzyme varies from 45000 to 60,000 (Guengerich and Liebler, 1985).

Cytochrome P450 exists in the oxidized state (Fe$^{3+}$). It is to this state of the cytochrome that the substrate will come and attach. The complex formed will accept an electron (via NADPH cyt. P450 reductase) resulting in the reduction of the cytochrome. Next molecular oxygen and then a second electron also attach to the complex. Through some yet unworked out steps, both electrons accepted by the complex get transferred to oxygen making it unstable and reactive. One atom of oxygen oxidizes the substrate while the second gets reduced to water. In this process cyt. P450, in its oxidized state, gets regenerated (Fig. 6).

The reduced Cyt. P450 (Fe$^{2+}$) and carbon monoxide combine to form a complex that gives a characteristic absorption maxima at 450 nm. It is from this property that Cyt. P450 gets its name.
Figure 6. Cytochrome P-450 electron transport systems and oxidation of a xenobiotic.
The mitochondrial P450 system contains an extra nonheme iron protein component—ferridoxin—which transfers electrons from NADPH ferredoxin reductase to Cyt. P450. (Fig. 7).

So far thirteen distinct forms of Cyt. P450 have been purified from rat liver microsomes (Nebert, 1987). It is estimated that the number of isoenzymes may go up to hundreds. These isoenzymes differ in substrate specificity, enzyme activity sites, absorption maxima of the CO-reduced cyt. complex, molecular weights, immunological properties, peptide maps of amino acid composition, induction properties, distribution and their regulatory control.

One form of Cyt. P450 is selectively induced by the carcinogen 3-methylcholanthrene (MCA). This isoenzyme also gives the characteristic absorption peak at 448 nm and not at 450 nm. Thus it is called Cyt. P448 or the aryl hydrocarbon hydroxylase (AHH). It is involved in the activation of polycyclic hydrocarbon (PAH). A cytosolic receptor of AHH has been identified (Nebert, 1989).

Cytochrome b₅

Cytochrome b₅ (Cyt. b₅) is a microsomal enzyme, found in conjunction with its reductase—NADH cytochrome b₅

22
Fig. 7 Electron transport in microsomal and mitochondrial monoxygenases. Abbreviations: FDX, Ferredoxin; FR, NADPH-ferredoxin reductase; Cyt.P450-Cytochrome P450 (From Pasanen and Pelkonen, 1990)
reductase. Both of these enzymes function together in the desaturation of fatty acids (Schenkman et al., 1976).

The relationship of cytochrome b\textsubscript{5} with the cyt. P450 system is still obscure. It may supply electrons to the P450 cycle (Schenkman et al., 1976).

Recently it has been shown that Cyt. P450 can bind with Cyt. b\textsubscript{5} to form a complex (Tamburnini et al., 1985). This binding enhances the substrate turnover rate of Cyt. P450 (Jansson et al., 1985; Tamburnini and Schenkman, 1987). Phosphorylation which is a form of post-transcriptional protein modification can inhibit this complex formation and P450 activity (Jansson et al., 1987). Cyt. b\textsubscript{5} is a competitive inhibitor of the phosphorylation of Cyt.P450 by protein kinase (Epstein, 1989). Maybe it competes for phosphorylation sites on Cyt. P450. Thus b\textsubscript{5} may have some regulatory function in Cyt. P450 activity.

GLUTATHIONE-S-TRANSFERASE (GST)

The Glutathione-S-transferases (E.C.2.5.1.18) are very important components of phase II metabolism. These enzymes, predominantly found in the cytosol, although a microsomal GST is now also known to exist (Morgenstern et al., 1982), catalyse the conjugation of electrophiles with the
nucleophilic thiolate anion of the endogenous tripeptide - glutathione.

The cytosolic GSTs are dimeric proteins and so far 12 subunits (M.W. \( \leq 25000 \)) have been identified in rat liver (Coles and Ketterer, 1990). These have been designated into 3 families which are \( \alpha \), \( \pi \) and \( \mu \) based on their primary structure (Coles and Ketterer, 1990). Combinations of identical subunits give rise to homodimers and different subunits of the same family to heterodimers. The GST coenzymes have overlapping substrate specificities.

Glutathione transferase activity resides in almost all the organs of the body and it is highest in the liver, testis, intestine, kidney and adrenal gland (Sipes and Gandolfi, 1986).

Different isoenzymes of GST are characteristic of a particular tissue. Also the occurrence of different forms of GST change in an organ specific manner during the change from fetal to adult state (Mannervik and Danielson, 1988). A sex related difference in hepatic expression of a specific enzyme form, apparently under testosterone control, has also been observed (Mannervik and Danielson, 1988). Some characteristic forms of GSTs are expressed in tumor tissue, thus making these enzymes good chemical markers (Coles and Ketterer, 1990).
Besides catalyzing conjugation reactions, GSTs have been postulated to act as intracellular carrier proteins and storage proteins (Mannervik and Danielson, 1988; Sipes and Gandolfi, 1986). Some form of GSTs have a peroxidase activity. These are the selenium independent glutathione peroxidases. These enzymes have low activity towards organic hydroperoxide and none at all towards \( \text{H}_2\text{O}_2 \) (Sun, Y., 1990).

Glutathione conjugates are excreted via the bile. These conjugates may also be converted into mercapturic acids, and excreted via the urine, through a number of enzymatic steps occurring mainly in the kidney (Sipes and Gandolfi, 1986) (Fig. 8).

Glutathione conjugation reactions do not always form nontoxic, easily excretable products, sometimes it can lead to the formation of toxic electrophiles. As for example, the conjugation of glutathione to ethylene dibromide which produces an unstable sulfur mustard (Pickel and Lu AYH, 1989). Several other cases are also known where the conjugation of alkyl and alkenyl halides with glutathione leads to the formation of toxic products (Peterson and Guengerich, 1988; Dekant et al., 1988).
Glutathione conjugation and mercapturic acid biosynthesis (From Sipes and Gandolfi, 1986).
Uridine Diphosphate Glucuronosyl Transferase (UDPGT)

UDPGT (E.C.2.4.1.17) catalyzes the conjugation reaction between UDP-glucuronic acid and xenobiotic electrophiles. Glucuronidation is an important conjugation reaction of phase II metabolism. It covers a wide range of substrates - alcohols, carboxylic acids, ketones, arylamines, tertiary amines, sulphonamides, aryl thiols etc. which can be conjugated with UDPGA. Conjugates of glucuronic acid are excreted via the bile or kidney depending on their molecular weights. Fig. 9 shows the structure of (UDP-GA).

UDPGT exist as several distinct isoenzymes. Three forms have been purified from rat liver. The exact number is still unknown.

UDPGTs are found in the endoplasmic reticulum of the liver, kidney, intestine, skin, brain and the spleen. Being a membrane bound enzyme, its close proximity to the phase I enzymes (the P450s) probably brings it in better contact with their reaction products, leading to an improvement in the detoxification process as a whole.

Glutathione

Glutathione (γ-glutamylcysteinylglycine) is a ubiquitous linear tripeptide in which the glutamyl moiety is
Fig. 9. Uridine-5'-diphospho-\(\alpha\)-D-glucuronic acid (UDP-GA)
bound via the γ-carboxyl group (Fig. 10). The thiol group is responsible for the chemical properties of the molecule. It exists in the thiol reduced (GSH) and disulfide oxidized (GSSG) forms. The main functional form is the reduced form - GSH.

Glutathione is the most important nonprotein thiol of cells. It has numerous diverse functions. It is probably best known for its conjugation - both spontaneous and enzyme catalyzed - with genotoxic electrophiles and the elimination of the GSH conjugate through the bile. The thiolate anion of GSH acts as an alternative nucleophilic site to the nucleophilic portion of DNA (Coles and Kettener, 1990).

It functions as an important redox buffer, by reacting reversibly with other thiol groups, thus affecting the redox state of protein - thiol functions (Gilbert, 1984). Since disulfide bond can change protein conformation this mechanism can regulate enzyme actions. Thus GSSG (oxidized form) is also called a 3rd messenger (Gilbert 1982).

GSH also has an important role to play in protein synthesis and degradation, formation of deoxyribonucleotide precursors of DNA, regulation of enzymes, protection of cells against reactive oxygen compounds and free radicals, and transportation of amino acids.
Fig. 10. Structure of glutathione (from DANIELSON 1987)
The highest concentration of GSH occurs in the liver, even though other organs contain high levels of GSH.

GSH depletion occurs under conditions of starvation and excess electrophilic burden on the body. Other factors which alter the GSH levels are oxidative stress, hormonal balance, growth and development (Kretzchmar, 1990).

GSH levels are the result of a dynamic process. It is continuously used up (GSTs and GPxs) and either gets oxidized to GSSG or broken down into its constituents. This process is balanced by the synthesis of GSH from its constituent amino acids. This involves two enzyme catalyzed steps:

1) The formation of the $\gamma$-glutamyl bond between glutamic acid and cysteine. This is catalyzed by the enzyme $\gamma$-glutamyl-cysteine synthetase (GCS).

   \[
   \text{L-Glu} + \text{L-Cys} + \text{ATP} \rightarrow \text{L-} \gamma \text{-Glu - L - Cys} + \text{ADP} + \text{Pi}
   \]

   This is the rate limiting step.

2) The formation of the peptide bond between $\gamma$-glutamyl cysteine and L-glycine which is catalysed by glutathione synthetase (GSHS).

   \[
   \text{L-} \gamma \text{-Glu - L - Cys} + \text{L - Gly} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{Pi}
   \]
GSH is also used as a cofactor in the enzyme catalyzed conversion of endogenous H$_2$O$_2$ to water. The enzyme catalyzing the reaction is glutathione peroxidase (GPx) and the reduced form of GSH is oxidized to GSSG. GSSG can be reduced back to the main functional form, GSH, by the NADPH dependent glutathione reductase (GR) (Fig. 11).

NADPH is taken mainly from the Pentose phosphate shunt.

**Glutathione Peroxidase (GPx)**

GPx (E.C.1.11.1.9) catalyses the conversion of H$_2$O$_2$ into water. In the reaction GSH gets oxidized to GSSG.

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O} \]

Depending on the selenium (Se) dependency, GPx can be divided into two forms.

1) Se-dependent GPx: These enzymes are tetramers with a molecular weight of $\approx 84,000$. They have very high activity towards H$_2$O$_2$ and other organic hydroperoxides.

2) Se-independent GPx: These are the GSTs. The enzymes are dimers with a mol. wt. of $\approx 50,000$. They have low activity towards organic hydroperoxides and none at all for H$_2$O$_2$. 

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Fig. 11  Overview on glutathione redox-cycle
GPxs are found in the mitochondria and cytosol. These are considered as primary antioxidants as they are directly involved in the elimination of active oxygen species.

**Glutathione Reductase (GR)**

GR (E.C.1.6.4.2) catalyzes the conversion of the oxidized form of glutathione (GSSG) to the reduced form (GSH). It uses NADPH which is mainly taken from the pentose phosphate shunt.

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{GSH} + \text{NADP}
\]

This enzyme has a molecular weight of \(\sim 120,000\). It has 2 subunits, each contains FAD at its active site. It has a similar distribution as GPx i.e. in the mitochondria and the cytosol.

GR is considered as a secondary antioxidant enzyme as it helps in detoxification of reactive \(O_2^*\) species by maintaining a steady supply of glutathione.

**CHEMOMODULATION OF CARCINOGENESIS**

The fact that spontaneous as well as induced tumor incidences can be modulated by several synthetic as well as naturally occurring compounds has been successfully exploited in the area of chemoprevention of cancer. The modulatory
action is achieved either by altering the drug metabolizing enzymes or by the direct actions on different steps of carcinogenesis in the target tissues. Direct actions might include the property of the modulatory substances either to make the target milieu more conducive for carcinogen action or act as scavengers of reactive carcinogenic species.

We have achieved the modulation of cervical carcinogenesis using a number of different synthetic as well as naturally occurring chemical compounds (Das et al., 1988; Rao and Hussain, 1988; Hussain et al., 1989; Hussain and Rao 1991).

AIMS AND OBJECTIVE

The possible cervical cancer risk among the users of oral or injectable contraceptive steroids elicits two main questions: (1) do these contraceptive steroids and their combinations induce cervical cancer among the users? (2) do these contraceptive steroids have any modulatory influence on the precancerous and cancerous lesions elicited by known or unknown carcinogens?

While the first problem is being adequately investigated, the second one has yet to receive sufficient epidemiological as well as experimental attention.
Besides this the drug metabolizing enzymes and the pathways responsible for biotransformation of xenobiotics have been found to be modulated or regulated by a large number of environmental and hormonal factors. Interaction of contraceptive steroids with liver drug metabolizing enzymes and in general with drug metabolism has been reported in a number of publications. These modulatory potentials of steroids on drug metabolizing enzymes might make an individual more prone or resistant to the action of different xenobiotic and endobiotic chemicals including carcinogen. However various animal species, different experimental approaches and various contraceptive steroids combinations have shown conflicting results.

Considering all these facts the present work was designed to assess

a) the modulatory influence of combined oral contraceptive pills, "OVRAL" (containing ethinylestradiol and norgestrel) and "NORACYCLINE" (containing ethinylestradiol and lynestrenol) on methylcholanthrene-induced carcinogenesis in the uterine cervix of mouse.

b) the modulatory influence of injectable contraceptive steroid "Medroxyprogesterone acetate" on methylcholanthrene-induced cervical carcinogenesis in mice.
c) the effect of combined oral contraceptive pills "OVRAL and NORACYCLINE" and injectable contraceptive steroid "Medroxyprogesterone acetate" on Phase I and Phase II drug metabolizing enzymes and acid soluble sulfhydryl level in the liver of mouse.

ORAL CONTRACEPTIVE PILLS: TYPES AND ACTIONS

Oral steroidal contraceptives are very common and popular method of birth control and are being used by as many as 60 million women globally (Hatcher et al., 1986). But despite the wide scale acceptance of oral contraceptive pill all over the world one of the major concerns in relation to its use has always been the possible stimulation of a preexisting malignancy or the development of a new one.

Depending upon the presence or absence of estrogen and progestogen component, the pills can be grouped into following types:

a) **Combined or Combination Pills**: These are combined estrogen and progestogen preparation;

b) **Sequential Preparations**: Contain estrogen only pills followed by combined estrogen and progestogen pills. But these preparations were withdrawn from use in 1970s because of the adverse side effect of estrogen alone;
c) **Triphasic and Biphasic Pills**: Contain estrogen and progestogens in varying combination for 21 days of use;

d) **Progestogen only Pills**: Contain only progestogen.

**Mechanism of Action**

Oral contraceptive steroids act primarily by inhibiting ovulation through suppressing the release of gonadotropins by inhibiting gonadotropin releasing hormone in hypothalamus. Besides this basic mechanism, the action of the combined preparations on other links in the reproductive chain also contributes in preventing pregnancy. These actions include:

i) Modification in the tubal contraction which affect the transport of an egg if ovulation did occur;

ii) Inhibition of implantation by affecting the endometrium;

iii) Progestogen influence which affect the capacitation of sperm during their transit through female genital tract.

Because of this doubly sure assurance the combination oral contraceptives are more effective than the progestogen
only pill. And only the 30 ug of estrogen in combination with a progestogen is as effective in preventing ovulation and altering cervical mucus as 50 ug of it.

Combined oral contraceptive pills used in the present study are:

a) **Ovral**, each containing 0.05 mg ethinylestradiol and 0.5 mg norgestrel (Fig. 12 and 13).

b) **Noracycline**, each containing 0.05 mg ethinylestradiol and 1 mg lynestrenol (Fig. 14).

**INJECTABLE CONTRACEPTIVE**

Medroxyprogesterone acetate (MPA) (Fig. 15), a progestin derived from the natural hormone progesterone, in its depot form is used as long acting injectable contraceptive in a number of developing as well as developed countries.

Although MPA was synthesized in 1954, its depot preparation was first tested as contraceptive by Tyler, Coutinho and others in early 1960's. The whole era of DMPA (Depot medroxyprogesterone acetate) has been highly controversial. Although it was used popularly in 1960s and 1970s in USA and by 1980's in 80 developed and developing
Fig: 12  ETHINYLESTRADIOL

Fig: 13  NORGESTREL
Fig. 14. LYNESTRENOL

Fig. 15 (MEDROXYPROGESTERONE ACETATE)
countries, in 1978 it was withdrawn from U.S. market following the pressure from Congress as well as certain public groups. The major concern was its carcinogenic action as shown by animal studies (WHO, 1982) and still the debate revolves around the possible carcinogenicity of injectable synthetic steroid.

Like oral contraceptive pill steroids, MPA also suppresses the release of LH and FSH by inhibiting gonadotropin-releasing hormone thereby inhibiting the ovulation.

AN OVERVIEW OF MOLECULAR ASPECTS OF STEROID HORMONE ACTION

The general idea about the steroid hormone action has been that it diffuses passively into the target cell, combines with an oligomeric cytoplasmic receptor, gets activated and then transported to the nucleus where it combines with DNA/chromatin. We find a general agreement in some of the basic events like passive entry into the cell, existence of an intracellular receptor with high ligand affinity and specificity, and the final site of action being chromatin but disagreement exists in the location and requirements of activation of receptors. On the basis of these major differences two models have been put forward which are as follows (Fig. 16).
Fig. 16. Intracellular events involved in steroid hormone action.

(A) Model in which the receptor is cytosolic and transfers to the nucleus after binding with steroid(s).

(B) Model in which unliganded receptor (θ) is in the nucleus

(From King, R. J. B., 1988).
1. In one model (Fig. 16A) the steroid receptor is cytosolic and after binding with the steroid it gets activated and transferred to the nucleus. The cytosolic '8S' receptor consists of a ligand binding unit and other units, one of which is a 90 KDa "heat shock" protein. Activation is shown here as involving dimerization of the ligand binding unit. This simplification of true events applies to estradiol receptor but not necessarily for other receptor classes. There is no agreement as to where the activation occurs.

2. In another model (Fig. 16B) nuclear localization of the unliganded receptors has been suggested. A conformational change occurs on binding steroid which may result in increased affinity for specific DNA sequences.

The '8S' cytosol receptor is a heterologous structure made up of both ligand binding and nonbinding subunits. Although the ligand binding units are different in the various steroid receptor classes, a 90 KDa heat shock protein is common to all such classes (Okret et al., 1985 and Renoir et al., 1986). This protein has been found to be present in all mammalian cells. The role of activation in changing receptors to a DNA binding form is not clear but
there is no doubt that a conformational change occurs in the
ligand binding unit that exposes a DNA binding domain on the
receptor.

DNA binding

Multiple regulatory units in DNA upstream of the mRNA
initiation site exist and complex interactions occur between
these units which may contribute to the specificity of
steroid hormone action. Hormone Responsive Element (HRE) is
one of these units which is termed as Steroid Response
Element (SRE) if the hormone is a steroid. SRE has features
of an enhancer of transcription (Chambon et al., 1984) where
the steroid receptor complex binds. The DNA receptor
interactions are accompanied by specific changes in the
structure of chromatin which may mediate the action of the
steroid by stimulating the binding of transcription factors
to promoter elements. Yamamota (1985) suggested that the
involvement of multiple enhancers in association with
promoter elements may form the basis for steroid control of
gene network. Thus, steroid receptor complexes function to
activate enhancers which alone or in combination with
additional enhancers may influence the interaction of
transcription factors with promoters and thereby regulate
the rate of gene transcription.
There are a number of determinants which have been attributed to biological activities of steroids. These are 1) Ligand availability; 2) Ligand specificity of receptors; 3) Availability of responsive genes; 4) specificity of steroid responsive element.

ORGANIZATION OF THE THESIS

The present thesis is divided into two main parts. PART A contains all the studies included in the Ph.D synopsis while PART B includes the other published/accepted research papers in the area of chemomodulation of carcinogenesis.

PART A

This Part Includes the Following 5 Chapters

CHAPTER I

Gives the Review of Literature on all the aspects undertaken in the present study.

CHAPTER II

Deals with the induction of cervical carcinogenesis by methylcholanthrene (MCA) in mice.

CHAPTER III

Covers the modulatory influence of oral contraceptive pills, containing both estrogen and progestogen components, on MCA-induced
cervical carcinogenesis in mouse. This Chapter has two parts:

IIIa Deals with the Oral contraceptive pill "Ovral"

IIIb Deals with the Oral Contraceptive pill "Noracycline"

CHAPTER IV Includes the influence of injectable contraceptive steroid "Medroxyprogesterone acetate" (MPA) on MCA-induced carcinogenesis in the uterine cervix of mouse.

CHAPTER V Covers the effect of oral contraceptive pills and injectable contraceptive steroid "Medroxy-progesterone acetate" (MPA) on Phase I and Phase II drug metabolizing enzymes and sulfhydryl level in liver of mouse. Chapter V also has two parts.

Va Deals with the effect of oral contraceptive pills "Ovral" and "Noracycline"

Vb Deals with the effect of MPA
PART B

This part includes published/accepted research papers in the area of chemomodulation of carcinogenesis:


