THEORETICAL INVESTIGATIONS ON THE ROLE OF MODIFIED DNA FOR CARCINOGENESIS BY POLYCYCLIC AROMATIC AMINES

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COURSES OFFERED	GRADES AWARDED
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R P Bajpai

(Prof. R.P.Bajpai)

Dedication

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to my

Dadu and Dimma

***

Science, like that Nature to which it belongs, is neither limited by time nor space; it belongs to the world and is of no country and of no age.

Sir Humphry Davy.

Contents

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Jayati Sengupta

(JAYATI SENGUPTA)

Chapter I

CHAPTER I

INTRODUCTION

I.1 The Cancer Phenomenon

The susceptibility of man to cancer as a leading cause of death among humans has made cancer research a prime field of study in today's world (Cairns 1981). This has naturally led to inquiry pertaining to the nature and aetiology of cancer itself. Cancer may be described as basically a disorder at the cellular level, where the structure, life and functioning of cells are radically altered from their normal counterparts. This is marked chiefly by a sharp departure from the normal phenomenon of growth and maturity followed by cell division or death associated with the life cycle of normal cells. Cancerous cells are characterised by an abnormal tendency to proliferate rapidly, not going through the normal phase of differentiation and attainment of maturity. If the phase of cell division were to be represented by a switch

which turns "on" every time the cell replicates, it would seem that, in cancerous cells, this switch is never turned "off", but maintained without allowing progression towards a well-differentiated maturity (Franks 1986).

This cellular disorder called cancer has its effects at the level of the tissue, the organ, the system and finally the living person or animal concerned. The breakdown in growth control leads to development of a mass of cells called a tumour. When cancerous cells acquire the potential for invading other tissues and organs (*metastasis*), the stage is set for spread of cancer to various parts of the body. The lethal effects of a cancer are manifested eventually through loss of vital organ function due to starvation, physical impediment of normal function, and resulting debility. About 10 to 20 per cent of human deaths are attributed to cancer-related causes (Nowell 1976).

Cancer may be described in terms of various successive phases : *initiation*, *promotion*, *tumour progression* and *metastasis*. The first step of cancer initiation consists of the creation of a potential cancer cell whose neoplastic nature still remains dormant, perhaps for years. The stage of promotion calls for the action of a promoting agent to convert the cell to a species capable of indefinite self-multiplication. The next stage of tumour progression, once started, ensures the growth of the

rapidly growing cell mass, and maintenance of the undifferentiated state of the constituent cells. Finally, when metastasis sets in, the tumour cells proceed to infiltrate into tissues or organs other than their own, depositing the seeds for further tumour development elsewhere in the living system (Fidler *et al* 1978).

While there is a line of thought which allows for the endogenous (spontaneous) origin of human cancers, in general, the aetiology of cancer is believed to be xenobiotic or external (Shields & Harris 1990). Thus, the causes of cancer emerge as being (a) **physical**, *eg*. X-rays, UV or gamma radiation, (b) **chemical**, whether organic or inorganic, and (c) **biological**, *viz*. the DNA and RNA tumour viruses. Radiation carcinogenesis is well-studied, being intimately related to the epidemiology of cancers in living victims of the nuclear holocausts at Hiroshima and Nagasaki during World War II (Kato & Schull 1982). Carcinogenesis by chemicals is linked to the prevalence and occurence of certain possible cancer-causing chemical agents in the human environment (Garner *et al* 1984). Cancer caused by viruses, although well-defined for many animal species, would not seem to be a leading cause of cancer in man, where only the human *papilloma* virus and the Epstein-Barr virus have been isolated as cancer-causing viral agents in man (Tooze ed. 1980).

An understanding of the origin and nature of human cancer paves the way for therapy. The world of medicine offers, in general, three major strategies for treatment of cancer - chemotherapy, surgery and irradiation - which may be used singly or in combination. Of late, a greater understanding of the immune system seems to bring the promise of an immunologically-based strategy for cancer therapy, wherein the cells of the body are "*taught*" or equipped, as it were, to fight cancer by themselves. Lastly, the possible healing effects of the mind or nervous system cannot be ruled out, which although not fully established yet, do seem plausible considering the enormous, untapped potential resources supplied by the brain and autonomic nervous system in combination with the immune system, as brought out through the newly-founded field of neuroimmunobiology.

I.2 Chemical Carcinogenesis

Chemicals in the human environment are believed to constitute the ultimate cause for upto 90 % of human cancer cases, by some estimates (Boyland 1968). These chemicals may be inorganic or organic (mostly the latter), and may be *natural* or *artificial* (synthetic). A large fraction of chemicals thought to be

carcinogenic in humans are the direct or indirect products of today's chemical industry (Tomatis *et al* 1978), or else associated with certain avoidable living habits or conditions (like smoking or chewing tobacco). Thus, the dictum that prevention is better than cure is particularly applicable to many (if not most) instances of human cancer through chemicals (Philippe 1995).

A great deal of time and effort has been devoted to investigating the carcinogenic effects of pure chemicals on animal systems, which long-term tests have been incorporated in monograph form (*eg.* the publications of the U.S. Dept. of Health and Human Services, from 1951) . In most instances, it emerges as fairly clear and conclusive that a given chemical may be labelled as definitely "*carcinogenic*" or "*non-carcinogenic*" in a given animal species, although some borderline cases do exist. What is particularly noteworthy is that these large numbers of chemicals testing positive for animal carcinogenesis may be conveniently grouped into distinct families or classes on the basis of chemical structure. This points to the importance of basic chemical structure as a determining factor for carcinogenicity of chemicals (Gilman & Swierenga 1984; Wagner 1984).

Such structurally related families of chemicals are characterised by the significantly large number of members testing positive for animal carcinogenicity (Ames *et al* 1973). Typical

examples of such families include polycyclic aromatic hydrocarbons, polycyclic aromatic amines, N-nitroso compounds of various kinds (*eg.* dialkylnitrosamines and nitrosoureas) (Preussman & Stewart 1984), and alkylating agents of various types (*eg.* alkyl esters) (Lawley 1984; Dipple & Moschel 1990). One corollary following from the importance of chemical homology for carcinogenicity is that similar or analogous biochemical mechanisms may be held to operate for the various members of a given family in the course of the biopathways for their carcinogenic activity. This allows for a greater or lesser uniformity of approach in dealing with the molecular basis for carcinogenic action.

In the long-term tests for animal carcinogenicity, a given chemical may be administered in a variety of administration routes and dosage schedules, which are not without their effects upon the nature and magnitude of the carcinogenic response. These routes include intravenous injection (iv), subcutaneous injection (sc), intraperitoneal injection (ip), topical application on the skin, inhalation, oral intake (po), besides others (Chankong *et al* 1985). Between the stage of carcinogen administration and the final stage of cancer manifestation, there lies an entire hierarchy and scheme of mechanisms and biochemical changes, many of which are now able to be defined or described fairly lucidly in

precise molecular terms (Jouanneau *et al* 1995).

In the past 25 years or so, a great deal of interest has been focussed on unravelling the molecular basis of chemical carcinogenesis. The findings of various noted groups now find ready incorporation into two distinct phases of the process of carcinogenesis by chemicals. *Phase 1* has been lent a clearcut definition through the well-accepted *Ultimate Carcinogen Theory* of chemical carcinogenesis, which serves to define the role played by the chemical carcinogen (and its bio-derivatives) before it interacts with the critical cellular macromolecules. *Phase 2* takes the cue from the *Somatic Mutation Theory* of cancer, and applies it to chemical carcinogenesis in terms of the precise chemical alterations the carcinogen initiates in the critical macromolecule, know known to be the nuclear DNA of somatic cells. These two key concepts comprise the pivotal basis around which revolves most of our current understanding of the molecular basis for chemical carcinogenesis, and are dealt with in detail in the next two Sections of this Chapter.

I.3 The Ultimate Carcinogen Theory

Once chemical carcinogenesis was firmly established as a well-documentated phenomenon, the question arose as to what mechanisms operate within the living system to enable the carcinogen to render its carcinogenic effect. Much of our present knowledge concerning *Phase 1* mentioned above stems from the revolutionary concepts introduced by Miller and Miller and co-workers in terms of the *Ultimate Carcinogen Theory* (Miller & Miller 1970; 1981; 1977). This theory, now widely accepted as fact, states that most (if not all) chemical carcinogens exert their carcinogenic effect through operating as a reactive electrophilic species *in vivo*, either by virtue of their own intrinsic electrophilicity, or by means of biochemical or spontaneous transformation *in vivo* to a reactive *electrophile*. The electrophilic reactant functions to chemically attack nucleophilic or basic sites on the critical cellular *macromolecules* (now believed to be DNA), and being the actual agent to trigger off the cancer initiation process, is termed as the ultimate carcinogen.

The ultimate carcinogen concept gave rise to the definitions of the parent, proximate and ultimate carcinogen species as participants along the carcinogenesis pathway for a given chemical

carcinogen. The term "*parent carcinogen*" refers to the chemical carcinogen in the form administered initially to the living system. When metabolic or spontaneous chemical transformation of the parent carcinogen occurs *in vivo*, the initial product(s) of these conversions are termed as the "*proximate carcinogen(s)*". The proximate carcinogen(s) may be further converted to a highly reactive electrophilic species which, being the actual cancer-initiating agent, is termed as the "*ultimate carcinogen*" species. The electrophilic nature of the putative ultimate carcinogen species was inferred from the nucleophilic character of the target sites attacked by chemical carcinogens *in vivo* and *in vitro*.

The ultimate carcinogen theory also engendered definitions of "*direct-acting*" and "*indirect-acting*" carcinogens. The former refer to chemical carcinogens which require no enzymatic or metabolic transformation *in vivo* to act as carcinogens, being sufficiently reactive in themselves, or being spontaneously decomposed in the cellular matrix to reactive electrophiles. The latter refer to those chemical carcinogens which cannot function as carcinogens unless they are "activated" metabolically through enzyme-mediated conversion to reactive electrophiles. Alkylating esters and most alkylnitrosamides furnish two examples of classes of *direct-acting* chemical carcinogens. *Indirect-acting*

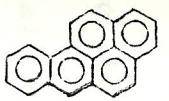
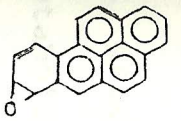
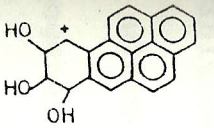
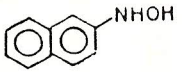
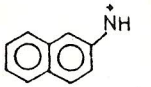
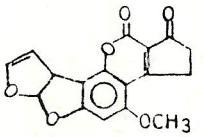
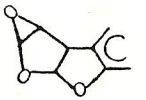
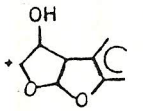
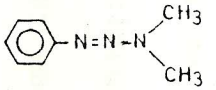
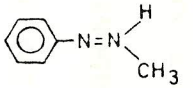
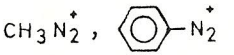
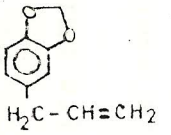
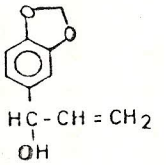
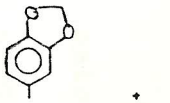
Family	Member	PaC structure	PrC structure	UC structure
Polycyclic aromatic hydrocarbons	Benzo(a)-pyrene			
Aromatic amines	2-Naphthyl-amine			
Aflatoxins	Aflatoxin B_1			
Aryltriazenes	3,3-dimethyl-1-phenyltriazene			
2,3 Propenyl-benzenes	Safrole			

Fig. I.1: Parent, Proximate and Ultimate carcinogen species for representative members of some families of chemical carcinogens (Duncan 1989)

carcinogenic families may be exemplified by the polycyclic aromatic hydrocarbons, polycyclic aromatic amines, most dialkylnitrosamines and aflatoxins.

The ultimate carcinogen concept is intimately related to carcinogenesis by aromatic amines and related compounds, since the initial groundwork done by the group of Miller and Miller was in fact performed on this family of carcinogens. The concept of the parent, proximate and ultimate carcinogens arose from studies on the metabolism of these compounds in various animal species, where the urinary metabolites furnished many clues. The present understanding of how carcinogenic aromatic amines are converted *in vivo* to their reactive ultimate carcinogen forms will be discussed in detail later (see Sec. III.2). By and by, it was found that these same ideas could well apply to other classes of chemical carcinogens as well, so that the *Ultimate Carcinogen Theory* eventually emerged as a unifying concept which provided a commonality of mechanistic understanding to chemical carcinogenesis.

Fig. I.1 presents some families of chemical carcinogens, some typical examples of each, and the structure of the corresponding proximate and ultimate carcinogen species. In most cases, it may be observed that the ultimate carcinogen is a positively charged species and obviously reactive as an electrophile.

I.4 The Somatic Mutation Theory

The role of critically altered macromolecules for carcinogenesis had long been postulated, but identification of the concerned macromolecule(s) came only with understanding of the role of DNA as the bearer of genetic information. Both genetic and epigenetic (Rhim *et al* 1971) mechanisms have been argued for carcinogenesis, with current evidence weighing heavily in favour of the former (Ts'O 1980). This has found expression as the Somatic Mutation Theory of Cancer, which had been stated in germinal form as long back as 1914 by Boveri (1914). This theory invokes the role of critically altered nuclear DNA of somatic cells for the initiation and progress of the cancerous state of the cell, which then, in principle, becomes inheritable from one cell generation to another. The critical alteration of DNA is performed in the case of chemical carcinogens (Marquardt 1979) by the reactive electrophilic ultimate carcinogen species, as the preceding Section has just described. Once altered, the process of DNA replication ensures that the cancerous information encoded in the modified DNA is preserved from generation to generation, thereby not requiring the continual presence of the carcinogen.

The somatic mutation theory of carcinogenesis as applied to chemicals has received much support from the following lines of evidence and observation as summarised below :

(1) DNA undergoes distinct chemical modification following administration of carcinogens *in vitro* and *in vivo*

(2) Chemical carcinogens in most cases also act as DNA mutagens

(3) The products of DNA modification by chemical carcinogens are known in many cases to possess "*promutagenic*" potential, *viz.* the ability to engender point mutations when present in templates for nucleic acid polymerases

(4) The discovery of oncogenes (reviewed by Teich 1986), which are DNA segments whose expression is intimately connected to various cellular and biochemical features of cancerous cells. The same applies to the discovery of tumour suppressor genes, which are DNA segments whose lack of expression contributes towards carcinogenesis (May & MAy 1995)

(5) The observed ability of chemical carcinogens in many cases to critically alter proto-oncogenic segments in the precise manner

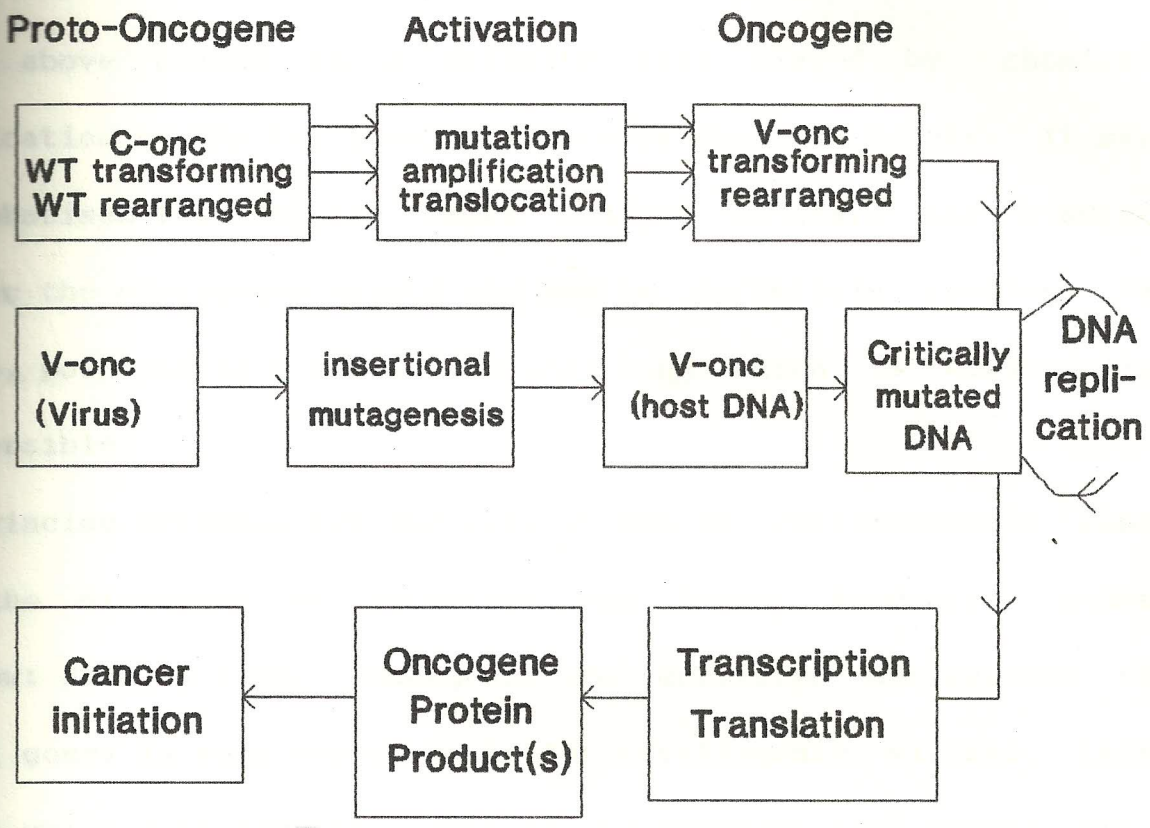


Fig. I.2: General concept of the Somatic Mutation Theory with some mechanisms of activation for proto-oncogenes (Duncan 1989)

required for activating it to the cancer-causing oncogenic form (Balmain & Brown 1988; Nagao *et al* 1993)

The above points to a definite role played by chemical modification of DNA for carcinogenesis by chemical agents. It may be emphasised that in the cases studied so far, the binding between the carcinogen moiety and DNA is definitely covalent in nature, pointing to a strong chemical bond which is essentially irreversible.

Convincing evidence for the role of DNA in carcinogenesis came with the discovery of oncogenes and tumour suppressor genes (review: Stanley 1995). Oncogenes are activated derivatives of normal genes in some cases, while for carcinogenic viruses, they may be quite different in structure and function from normal DNA. The former are called *c-onc* genes, while the latter are termed as *v-onc* genes. The *c-onc* genes are present in latent form in normal DNA, and are called proto-oncogenes. The mechanisms for activating proto-oncogenes to their carcinogenically active form include point mutations, gene allocations and gene amplifications, which are portrayed in Fig. I.2. For the v-onc genes, a reverse transcriptase enzyme serves to incorporate the viral DNA into the host DNA by transfection.

I.5 Aromatic Amines as Carcinogens

The broad term "*aromatic amine*" has been used to refer to any compound with an amino or imino group attached to an aromatic ring. This classification embraces those compounds which are truly primary aromatic amines, as well as a number of their derivatives which include acylated, esterified, and other secondarily substituted derivatives, so that the term "*N-substituted aromatic compounds*" might seem more appropriate for the class as a whole. This includes also hydroxamates and amides, besides amine oxides and quaternised derivatives. This class of compounds has come into prominence since the last century due to their importance for manufacture of azo dyes, plastics and pharmaceutical drugs (Singer & Grunberger 1983).

The first evidence suggesting the carcinogenicity of aromatic amines was noticed by Rehn (1895) who reported a correlation between human cancer incidence and exposure to aromatic amines. Subsequent epidemiological studies by Case and co-workers (1954) convincingly demonstrated a bladder cancer hazard for workers in the azo dye industry, being linked particularly to exposure to 2-naphthylamine and benzidine. Another aromatic amine, 4-aminobiphenylamine (Mellik *et al* 1955), has also been shown to be a definite bladder carcinogen in humans. This work has

established many aromatic amines as proven human carcinogens, albeit not through direct testing using human subjects, but through the weight of epidemiological evidence.

The animal carcinogenicity of aromatic amines was first shown by Hueper *et al* (1938), being demonstrated in dogs for 2-naphthylamine through injection and administration through the diet. Since then, a large number of aromatic amines and related compounds have entered the ranks of chemicals proven to be carcinogens in various animal species, which information is compiled in standard monographs. In higher mammals, the incidence of cancer is largely found to occur in the bladder, as is the case with man.

In fact, aromatic amine carcinogens do not generally induce tumours at the site of administration, which is in line with their character as indirect-acting carcinogens. Common target organs for tumourigenesis include the urinary bladder, liver and intestine. These observations have led to the inference that metabolic transformation and transport play an important role for aromatic amine carcinogenesis. This formed the basis for the important work of Miller and Miller and their co-workers, who discovered the role of metabolism for aromatic amine carcinogenesis, thereby laying the foundations for the *ultimate carcinogen theory* of chemical carcinogenesis. The role of the

ultimate carcinogen species in aromatic amine carcinogenesis is further dealt with in Chapter Three.

The role of altered DNA for aromatic amine carcinogenesis also seems to be receiving increasingly clearer confirmation with the discovery of the adducts formed between DNA bases and aromatic amine metabolites *in vitro* and *in vivo*. Further evidence pointing along this line is provided by the role of proto-oncogenes critically mutated following administration of aromatic and heterocyclic amine carcinogens (Nagao *et al* 1993). The application of the *somatic mutation concept* to aromatic amine carcinogenesis is further dealt with in detail in Chapter Four.

The role played by chemical structure in determining carcinogenicity of aromatic amines has been well-studied. Simple compounds with only one aromatic ring appear to be non-carcinogenic or at most weakly carcinogenic. This is the case for aniline and acetanilide, both non-carcinogenic in animals. The methyl substituted *o*-toluidine yields a weak carcinogen. The presence of two or more aromatic rings, whether fused or separate, seems to be one factor linked to carcinogenicity. Thus 2-naphthylamine, 4-aminobiphenyl, 2-aminofluorene and benzidine are representative of this case as proven carcinogens (Bonser 1943; Walpole *et al* 1952; Spitz *et al* 1950; Cox *et al* 1947; Wilson *et al* 1947). However, these simple structure-activity relationships do not furnish an infallible criterion for presence or absence of carcinogenicity.