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

BY

JAYATI SENGUPTA

INSTITUTE OF SELF ORGANISING SYSTEMS AND BIOPHYSICS

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पूर्वोत्तर पर्वतीय विश्वविद्यालय
पू० प० विवि० परिसर, शिलांग-७९३०२२ (मेघालय)
Phone :
Grams : NEHU
North-Eastern Hill University
NEHU Campus, Shillong - 793022 (Meghalaya)

INSTITUTE OF SELF ORGANISING SYSTEMS & BIOPHYSICS

- Pre-Ph.D. Course Grade Certificate -

This is to certify that Ms. JAYATI SENGUPTA secured the grades mentioned below in the following Pre-Ph.D. courses:

COURSES OFFERED	GRADES AWARDED
1. Self-Organising Systems	A
2. Methods of Science	A
3. Living State and Biophysics of Living Organisms	A
4. Computer Applications to Biology	A

R P Bajpai

(Prof. R.P.Bajpai)

पूर्वोत्तर पर्वतीय विश्वविद्यालय

बिजनी परिसर, शिलांग-७९३००३ (मेघालय)

Phone :
Grams : NEHU

North-Eastern Hill University

BIJNI Complex, Shillong - 793003 (Meghalaya)

DEPARTMENT OF CHEMISTRY

- CERTIFICATE FROM SUPERVISOR -

This is to certify that the thesis entitled "Theoretical Investigation on the Role of Modified DNA for Carcinogenesis by Polycyclic Aromatic Amines", submitted to North Eastern Hill University, Shillong, by Ms. Jayati Sengupta in fulfilment of the requirements for the degree of Doctor of Philosophy embodies the record of original research work carried out by her under my supervision. She has been duly registered and the thesis submitted is worthy of being considered for the award of the Ph.D. degree.

This work has not been submitted for any other degree to any other University or Institution.

16/5/96

(Dr. R.H.Duncan Lyngdoh)

Supervisor

Dedication

***

to my

Badu and Zimma

***

Science, like that Nature to which it belongs, is common limited by time nor space; it belongs to the world and to all the country and all the age.

Sir Humphry Davy.

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Chapter 1

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*

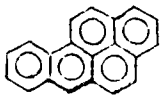
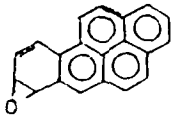
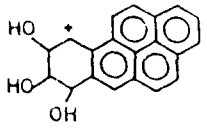
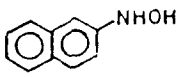
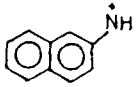
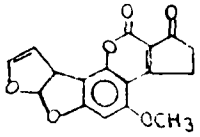
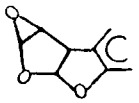
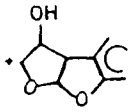
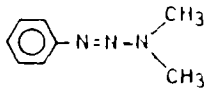
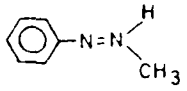
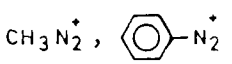
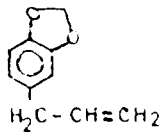
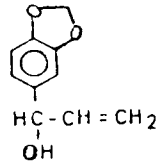
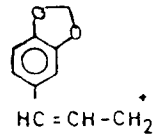
Family	Member	PaC structure	PrC structure	UC structure
Polycyclic aromatic hydrocarbons	Benzo(a)-pyrene			
Aromatic amines	2-Naphthyl-amine			
Aflatoxins	Aflatoxin B_1			
Aryltriazenes	3,3-dimethyl-1-phenyltriazene			
2,3 Propenyl-benzenes	Safrole			

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

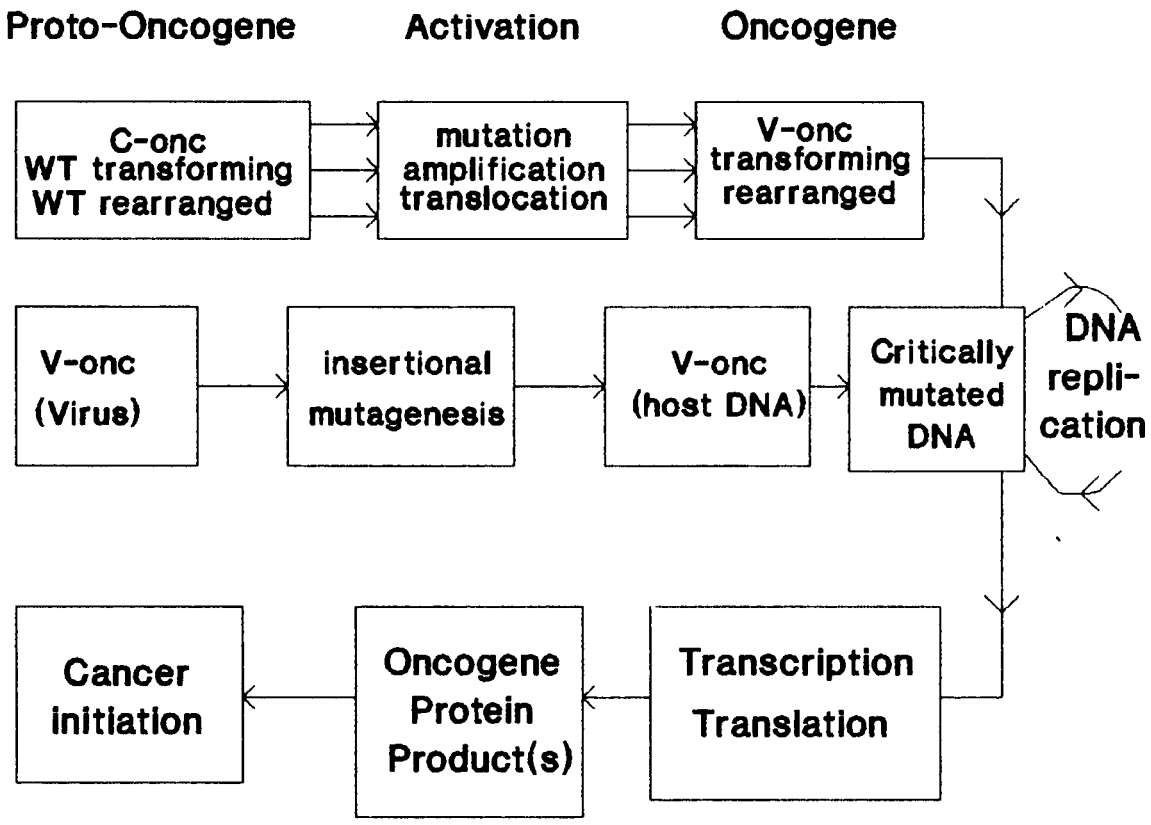


Fig. I.2: General concept of the Somatic Mutation Theory with some mechanisms of activation for proto-oncogenes (Duncan 1989)

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.

Chapter II

CHAPTER II

APPROACHES TO INVESTIGATION

II.1 Understanding Cancer in Molecular Terms

This dissertation primarily concerns the study of aromatic amine carcinogenesis using the methods of molecular quantum mechanics. A formal prerequisite to the investigation of a complex biological phenomenon like cancer by the methods of chemistry is that the phenomenon be expressed or reduced to precise molecular terms. This may be done either by drawing from the proven results of experiment on the molecular mechanisms underlying the phenonomen, or else through the proposal of a viable hypothesis which can express carcinogenesis in molecular language. Molecular quantum mechanics is the basic tool of investigation for this dissertation, through use of semiempirical molecular orbital theory. Such a tool can be meaningfully applied only when the phenomenon itself lends itself amenable to investigation by

methods involving the concepts of molecular structure, function and interaction.

Happily, the situation now is not as formidable as it once seemed. Even barely two or three decades ago, a venture of the kind envisaged in this dissertation might have seemed a hopeless task. Fortunately for the quantum chemist or user of molecular orbital methods, the picture now emerging is that the molecular basis of carcinogenesis is, at least in many of its crucial aspects, quite reducible to precise molecular terms. This is due to the multi-pronged efforts of experimentalists in various groups the world over, which together have contributed immensely to our present understanding of cancer and chemical carcinogenesis at the molecular level.

Fundamental to our current understanding of the molecular basis of chemical carcinogenesis are the revolutionary ideas introduced by the *Ultimate Carcinogen Theory* and the *Somatic Mutation Theory* of cancer. These two concepts furnish the key pivots around which revolves our present knowledge of the molecular basis for chemical carcinogenesis. Basing upon these twin theories, the quantum of work on chemical carcinogenesis done by chemists, biochemists and molecular biologists acquires a meaningful and cohesive character. Briefly stated, a general molecular mechanism for chemical carcinogenesis may be formulated as based upon this twin

foundation, which consists of the following basic steps (Duncan 1989) :

(1) Administration of the chemical carcinogen to the bio-system, followed by transport *in vivo* to the site of initial interaction with the cellular macromolecules

(2) If the compound is *direct-acting*, go straight to step (4)

(3) If the compound is *indirect-acting*, metabolism of an oxidative nature occurs, whereby the parent carcinogen is converted to its proximate carcinogenic form through enzymatic oxidation

(4) The formation of the ultimate carcinogen follows, which occurs spontaneously from the parent carcinogen if it is direct-acting, but from the proximate carcinogen if indirect-acting

(5) The ultimate carcinogen then attacks critical nucleophilic sites on the nuclear DNA of somatic cells of the target organ, which sites occur on appropriate points or segments of the proto-ongogenic sequence present in the host DNA

(6) The critically modified DNA acquires a "*pro-mutagenic*" character through a variety of ways (depending upon the type of carcinogen family), but ultimately leading to the induction of the critical DNA mutation(s) *via* the process of DNA replication

(7) The critical DNA mutation(s) serve to "*activate*" the proto-oncogene, which becomes converted to its carcinogenically active oncogenic counterpart, which activation may occur through point mutations, chromosomal translocations or gene amplifications

(8) The activated oncogene is then expressed through synthesis of the oncoproteins, which go on to participate in the cellular biology of the cancerous cell, lending it the peculiar characteristics of neoplastic transformation.

Thus we see that carcinogenesis may be understood at any level along a hierarchy of levels of understanding, the most fundamental being the quantum chemical description of the molecular details of the phenomenon. This hierarchy of levels of understanding (Haorah 1995) include the following :

(a) *Epidemiological* understanding of cancer, directed towards entire populations

(b) *Clinical* understanding of cancer, directed towards human individuals

(c) *Histological* understanding of cancer, directed towards organs or tissues of affected hosts

(d) *Cytological* understanding of cancer, at the level of cells

(e) *Molecular biological* understanding of cancer, concerning the role of macromolecules in carcinogenesis

(f) *Toxicological* understanding of cancer, concerning the interactions between carcinogen and living system components

(g) *Bioorganic* understanding of cancer, analysing the mechanism of cancer in terms of molecular structure and interaction

(h) *Quantum chemical* understanding of cancer, applying the quantum mechanical theory of molecular structure and interaction to the phenomenon of cancer, being the most fundamental description of the process

II.2 Experimental Approaches to Carcinogenesis

This section deals with a brief review of some approaches adopted by experimental science to investigate the phenomenon of chemical carcinogenesis, especially with regard to aromatic amines. These may be summarised as follows :

(1) *Determination of carcinogenicity* : For most families of chemical carcinogens, this has formed the major part of time and effort expended from the point of view of cancer research. This line of work focusses primarily on the determination of the presence or absence of carcinogenicity of a particular chemical. By far the most definitive test used is the long-term laboratory

animal assay, where a variety of animal species, chiefly rodents, are subjected to exposure to the compound over various periods of time. The administration regime may involve single-dose, multiple-dose, chronic exposure and life-long schedules, as well as multigeneration exposure spans. The most comprehensive compilation of these approaches is represented by the *Survey of Chemicals Tested for Carcinogenicity*, as brought out by the United States Department of Health and Human Services, in a series of valuable monographs (from vol.1, 1951).

This approach hinges upon the choice of a suitable definition of "*carcinogenicity*". By and large, the chronic exposure regimes with small doses may be regarded as the best simulation of what could be an environmental exposure. The carcinogenic response is determined by the appearance of tumours as compared to controls. As such, a chemical may be classified as carcinogenic, non-carcinogenic or as a possibly borderline case (when the maximum dose tolerated is not administered). Note that the response may be subject to the animal species tested, where some species seem resistant to the carcinogenic effect of a chemical, while others are not. For example, *Xenopus laevis* does not register a positive response to direct-acting N-nitroso carcinogens, while mammalian species do (Preussmann & Stewart 1984). This then raises the question of how these animal tests may be extrapolated to man.

Long-term animal tests have been performed for all the major families of carcinogenic compounds. Apart from the overall survey quoted above, individual monographs and articles have been devoted to the various families. Representative oncogenesis tests for alkylating agents as a class have been reviewed by Lawley (1976). N-nitroso compounds have also received much attention by way of a number of reviews and monographs (Preussmann & Stewart 1984; Preussmann & Eisenbrand 1984). Polycyclic aromatic hydrocarbon compounds and their carcinogenic effects have been summarised in reviews by Dipple and others (1984), while polycyclic aromatic amines and related compounds have figured in reviews by Garner *et al* 1984).

The overall inferences from these compilations is that, by and large, chemical carcinogens tend to form families of homologously or structurally related compounds, not all of which may be carcinogenic though (IARC Monographs 1980).

Because of the large cost and time involved in these long-term animal tests, a number of cheaper short-term alternatives not using intact animals have been proposed. All of these depend upon a particular model or understanding of the carcinogenic process, and interpretation may be made accordingly. Most of them stem from the somatic mutation concept, and therefore are linked to the manifestation of a mutagenic response. The Ames *Salmonella* mutant

reversion test has been much used to compare with the results of long-term carcinogenicity trials, and on the whole points to a favourable link between the two (Ames *et al* 1973; Josephy 1989). The use of *in vitro* tissue culture tests (*in vitro* carcinogenesis) involves the neoplastic transformation of cells in culture, which have included mammalian and human cell lines too (Heidelberger 1977; Kakunaga 1977). Another possible test is the direct oncogenic transformation of known proto-oncogenic DNA sequences *in vitro* by administration of the carcinogen (Cho *et al* 1992). The predictive value of these tests in combination has been statistically analysed (Chankong *et al* 1985) using a Bayesian algorithm incorporating a battery of such tests.

(2) *Evaluation of human carcinogenic risk* : Since living humans cannot serve as subjects for carcinogenicity testings, whatever evaluation concerning human carcinogenic risk of a chemical has to be obtained from epidemiological surveys on a statistical basis (Hayashi & Sugimura 1994). This involves examination of incidences of cancer among a population susceptible to risk by virtue of uncommon exposure to the chemical(s), the result of which is then compared with the population at large. The noteworthy survey of Case and co-workers (1954) on bladder cancer incidence among dye manufacture workers was what confirmed the

human carcinogenic potential of several aromatic amines. A total of about 25 different chemical substances have been earmarked as carcinogenic for humans on this basis (Wigley 1986). A strategy has been suggested to use the mode of action of chemical carcinogens as a guide for assessment of carcinogenic risk (Butterworth *et al* 1995).

(3) *Salient features of chemical carcinogenicity tests* : Along with the determination of the presence or absence of carcinogenic effect as such on animals, corollary features include determination of relative carcinogenic potency (or tumour-inducing power) as well as the physiological study of the target organs of the animal particularly affected by cancer. Such studies obviously involve only carcinogenically active compounds. Comparison of relative carcinogenic potency may be made among chemicals of different families, or within the same family. To ensure equability and self-consistency of the results, the cancer-inducing process has to be conducted under similar or identical conditions, employing the same species or strain, the same dosage regime and the same dietary and living conditions. Carcinogenic potency indices have been formulated in various ways, including the Iball index (1939) used for polycyclic aromatic hydrocarbons (Herndon 1974), the Wishnok-Archer dose-response

index (1976) used for N-nitroso compounds, and the use of a varying number of plus signs (+) to roughly indicate varying carcinogenic strengths of the compounds. Besides the *in vivo* long-term tests, the somatic mutation concept has been invoked to assess genotoxic potential through cell lines in culture and through the Ames mutant reversion test, the results of which, however, do not always compare well with the results of long-term tests.

Likewise, the study of the target organs particularly effected carcinogenically has furnished a fascinating field of work (Lutz *et al* 1994). Among the various sub-groups of N-nitroso carcinogens, a remarkable specifity of choice of the target organ has been noted, which effects have been treated on a mechanistic basis recently (Haorah 1995). For aromatic amines, the specific recurrence of bladder cancers in dogs and in humans has been a noteworthy finding (Hueper *et al* 1938), for which plausible rationalisations have been provided.

(4) *Elucidation of the molecular basis of cancer* : This thesis is primarily concerned with studying aromatic amine carcinogenesis at the molecular level, for which an adequately clear and well-defined basis should present itself, either through the results of experiment or through a plausible hypothesis. This may

be described as the study of chemical carcinogenesis at its most fundamental and irreducible level. The molecular basis of cancer can, in turn, be utilised to furnish rationalisations and predictions of the carcinogenesis phenomenon at higher levels along the hierarchy of levels of understanding of the phenomenon, so that the macroscopic aspects of the phenomenon may be described in terms of the microscopic.

It has turned out that, by and large, the description of chemical carcinogenesis in molecular terms fits in well with the *ultimate carcinogen theory* and the *somatic mutation theory*. These two concepts thereby provide two distinct stages of the carcinogenesis phenomenon, of which the first may be understood in terms of simple chemistry, while the latter requires a good understanding of molecular biology.

Alkylating agents and N-nitroso compounds are perhaps the chemical families for which the molecular basis of carcinogenic action has been defined most lucidly for both stages. The metabolism and ultimate carcinogen generation of polycyclic aromatic hydrocarbons is now fairly well-understood, and many covalently bound carcinogen-DNA adducts have been characterised. The phases involving and following critical DNA mutation by hydrocarbon metabolites have, however, so far eluded unambiguous definition. The case is much the same for polycyclic aromatic

amines, where the metabolites, the putative ultimate carcinogen species and the carcinogen-DNA adducts have been discovered or their identities been inferred from experimental results, while the steps following DNA modification are still not too clear. The quantum of experimental work on the molecular basis of aromatic amine carcinogenesis will be described in greater detail in the beginning sections of the remaining chapters of this thesis.

II.3 Theoretical Approaches to Carcinogenesis

It has been stressed that an adequate understanding of cancer in precise molecular terms is a prerequisite to formal treatment using theories of chemical bonding and structure. Some efforts, of course, have been made towards achieving a clearer understanding of the phenomenon in its macroscopic and physiological aspects without reducing it to molecular terms. By and large, however, the bulk of theoretical work on chemical carcinogenesis has been pursued having the molecular aspects in mind. The chemical theoretician's approach to the study of chemical carcinogenesis has in general followed two distinct viewpoints :

(1) *Classical structure-activity relationships* : These constitute the establishment of connections between classical chemical structure and certain salient features of carcinogenicity without recourse to detailed and more expensive molecular orbital calculations. Features of the chemical structure deemed relevant for cancer are subjected to numerical evaluation by various means, and the results used in attempts to screen for carcinogenic activity, to gauge for potency, or to relate to choice of the target organ. Usually, the focus is upon the parent carcinogen species, so that precise definition of the successive steps along the carcinogenesis pathway is not considered explicitly, especially at the stages involving and following critical DNA modification. The methods of evaluation for such indices include simple molecular weight or size determination (Druckrey *et al* 1967), determination of appropriate bond lengths, and the use of molecular topology and graph theory (Seybold 1983).

(2) *Quantum chemical approaches* : With the advent of quantum mechanics, the description of molecular structure and interaction followed the concept of the wave-particle duality of electrons existing within a nuclear framework. Quantum chemical treatment of molecules has followed two distinct lines of approach : (a) the *valence-bond* formulation of Pauling, and (b) the *molecular orbital*

description pioneered by Mulliken, of which the latter has received by far the greater attention and application. To this may be added the perturbational treatment, which is of direct application to molecules interacting at non-covalently bonded internuclear distances. With the molecular basis of chemical carcinogenesis becoming increasingly more lucid over the past three or four decades, the phenomenon has lent itself correspondingly more and more amenable to quantum chemical treatment. The various successive stages of the biochemical carcinogenesis pathway have been studied this way, using mainly the molecular orbital formalism in its varying levels of rigour. These range from the simple Huckel molecular orbital theory for π electrons, and then through the semiempirical valence-electron approximations, to the *ab initio* level with or without explicit consideration of electron correlation.

Primary attention has been devoted to arriving at effective screening criteria for carcinogenic activity with the use of appropriate indices derived from molecular orbital theory. Such indices include relevant bond orders, atomic charges, delocalisation energies, activation energies and thermodynamic stability criteria. Other features of the carcinogenesis phenomenon handled theoretically include the rationalisation of potency variations, the key molecular aspects of DNA modification

and of mutagenesis, as well as the specific choice of repair mechanisms for different types of modified DNA constituents.

Initial interest in theoretical treatment is was sparked off by the well-known *K-L-region theory* of Pullman and Pullman (1954,1955) which handled carcinogenicity of polycyclic aromatic hydrocarbons with the help of molecular indices derived from the simple Huckel theory. The numerical values of these bond indices served to screen fairly well for carcinogenic activity among a large number of members of this family. Later years saw the further development of this basic theory, handled by molecular orbital methods of increasing vigour. While the *K-L-region theory* focussed explicitly only on the parent hydrocarbon, the later "bay-region" theory incorporated the metabolites too (Jerina *et al* 1977).

The comparatively simple and clearly understood molecular basis proposed for carcinogenesis by N-nitroso compounds and alkylating agents has prompted a large body of molecular orbital studies on these groups of chemicals. Duncan (1989) used the semiempirical INDO SCF MO method to study the successive stages of the carcinogenesis biopathway for N-nitroso compounds, which included the parent and proximate carcinogens, the ultimate carcinogen, the carcinogen-DNA adducts, the induction of point mutations, and repair of the modified DNA. Ford and Scribner used the MNDO SCF

MO method to probe into the alkylation of DNA and the procarcinogenic role of the adducts formed thus (1983). Aberrant base-pairing schemes leading to point mutations have also received some theoretical attention (Venkateswarlu & Lyngdoh 1995).

The fairly abundant amount of theoretical work performed on aromatic amine carcinogenesis will be reviewed stage by stage in the remaining chapters of this dissertation.

II.4 Formulation of the Research Problem

In consideration of the formidable difficulties encountered in even proposing a plausible and lucid molecular basis for carcinogenesis by aromatic amines, it is incumbent upon the researcher to resort to a number of simplifying assumptions in order to arrive at a workable basis for framing a suitable theoretical approach to the study of carcinogenesis by aromatic amines and related compounds. These include the following :

1. The semiempirical MNDO SCF molecular orbital method is used for providing the wave-functions, optimised structures and all molecular properties for every phase of the phenomenon studied in this dissertation. This method is suitable for medium-sized to

large molecular systems, being reasonably accurate when compared with more sophisticated and rigorous *ab initio* methods.

2. The gas phase model is used for studying all molecular structures and interactions, even though these occur in the solvent phase *in vivo*, since calculations in solvent phase are considerably more time-consuming than those in gas phase, and especially so for the large systems envisaged here.

3. The metabolism of aromatic amines is studied here through a very simplistic model which replaces the oxidised heme moiety of the cytochrome oxidase enzyme system by a single triplet oxygen atom, as has been done by Loew *et al* (1983) for metabolism of nitrosamines and other xenobiotics.

4. The mutational aspects of aromatic amine carcinogenesis are here restricted to point mutations only (such activation of proto-oncogenes being very well-known), and it is further supposed that these point mutations consist of base substitutions or frame-shifts engendered through creation of abasic sites.

The following points are also to be noted :

1. Since the precise molecular mechanisms for aromatic amine carcinogenesis are still not clear on many counts, it remains upon

the researcher to hypothesise suitable models for the process at its various stages which satisfy the following criteria :

(a) The model should be clearly defined at the molecular level

(b) The model should be accessible to study by the MNDO SCF MO methodology

(c) The model should draw all it can from available experimental data and observations

(d) The model should be sufficient to furnish rationalisations for the pertinent aspects of the phenomenon under study

(e) The model should be dependable enough to provide reasonable predictions concerning aspects yet unknown to science

2. When focussing upon a particular pertinent aspect of the carcinogenesis phenomenon, all information concerning should be collected together to see how far the above mentioned model can serve to provide coherent rationalisations and explanations for these aspects on the basis of the calculations performed.

3. With the absence of candid information on many experimental aspects of the phenomenon, one has also to lay recourse to the furnishing of tenable predictions concerning these aspects, which yet await fulfilment following further work in the future.

Bearing these in mind, the research problem may be formulated in general terms as follows :

1. The carcinogenesis pathway is hypothesised to proceed *via* a particular well-defined molecular mechanism, which includes (a) metabolism of the parent amine yielding the *ultimate carcinogen*, (b) modification of DNA bases by the ultimate carcinogen, (c) induction of point mutations by the modified DNA *via* creation of abasic sites, and (d) resultant *proto-oncogene* activation. Corresponding steps with a deactivating or detoxifying aspect include loss or repair of modified DNA. The details concerning these steps are procured from experimental data, as well as from judicious hypothesising.

2. For each phase or stage along the above postulated pathway, the pertinent aspects worth considering are isolated for study. These include the main feature of each phase (*eg*. the product distribution for the DNA modification reaction stage), along with some corollary or secondary aspects such as structure, conformation or minor aspects of reactivity.

3. The gas phase models are then constructed for each phase of the carcinogenesis pathway in a manner appropriate for handling through the use of the MNDO SCF method.

4. These models then serve as the basis for performing molecular orbital calculations at the semiempirical MNDO level, from the results of which (the wave-function, optimised structure and calculated molecular properties), the relevant rationalisations or predictions can be drawn up and applied accordingly.

The particular manner in which the above general formulation is applied to the successive stages is discussed in detail in the each of the Chapters dealing with these stages. Chapter Three deals with the metabolism of the parent amines to reactive metabolites, in particular touching on the generation of the putative ultimate carcinogen species. Chapter Four then deals with the modification of DNA constituents by the ultimate carcinogen species. Chapter Five concerns pertinent structural changes following DNA modification which could have bearing on the molecular biology of aromatic amine carcinogenesis. Finally Chapter Six handles the loss of the base or carcinogen moieties from the modified DNA constituents, with its implications for *in vivo* stability, mutagenesis and even repair.

Chapter III

CHAPTER III

AROMATIC AMINE CARCINOGENS AND THEIR METABOLITES

III.1 Metabolic Activation of Carcinogenic Aromatic Amines

The postulates of the well-known ultimate carcinogen theory of chemical carcinogenesis invoke the creation *in vivo* of a reactive electrophilic species capable of readily attacking and modifying DNA at critical sites. This theory permits the broad classification of chemical carcinogens into the indirect-acting and direct-acting types, where the former require enzymatic transformation of chemical structure to act as carcinogens, while the latter require no enzyme mediation. The phenomenon of indirect-acting chemical carcinogenesis further calls for definition of the parent, proximate and ultimate carcinogen species, which has been explained earlier (Sec. 1.3). It is noteworthy that the pioneer studies of Miller and Miller and other

workers on the metabolism of aromatic amines in mammals was what first gave birth to the ultimate carcinogen concept, which by generalisation to almost all families of chemical carcinogens has now attained stature as the ultimate carcinogen theory. Fig. III.1 depicts the application of the ultimate carcinogen theory to carcinogenesis by aromatic amines (Ford & Herman 1992), where the proximate carcinogen metabolite are supposed to be the N-hydroxy amine and its esterification product, while the ultimate carcinogen is identified as the arylnitrenium ion.

Being classified as indirect-acting carcinogens, the large class of aromatic amines and related compounds are believed to exert their xenobiotic, genotoxic, and carcinogenic activity not in the form in which they are administered (the parent carcinogen), but only following *in vivo* metabolic (enzyme-mediated) transformation to a chemically more reactive species. The parent carcinogen here is initially metabolised to a fairly stable species called the proximate carcinogen (which later converts spontaneously to the putative reactive ultimate carcinogen). Elaborate and thorough survey of the metabolism of polycyclic aromatic amines in mammals was first carried out in the context of their carcinogenic effects in dogs through isolation and identification of the urinary metabolites of various carcinogenic members of the class (Miller 1970; Kadlubar *et al* 1977). Apparently, the main enzymatic transformations involved were of an oxidative nature, being

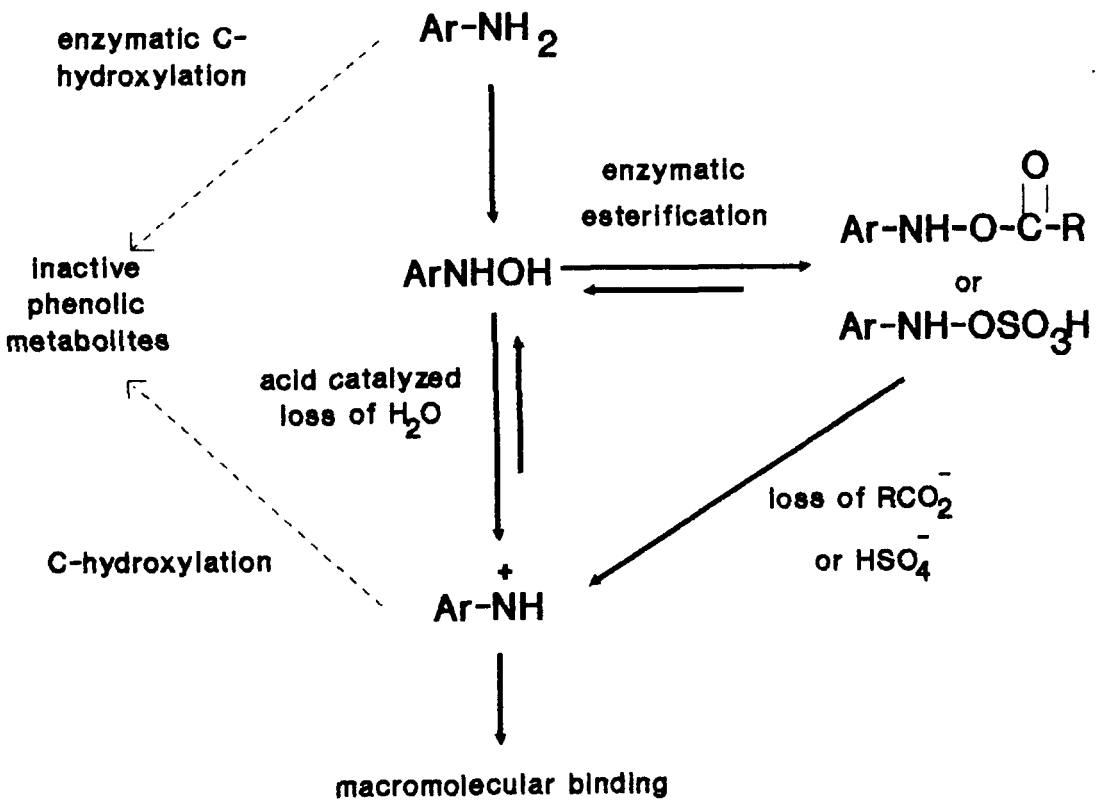


Fig. III.1: Metabolic activation and deactivation pathways for aromatic amines

mediated by enzymes of the *cytochrome P-450* oxidase series (Mcmanus 1989). Oxidation of primary and secondary aromatic amines *in vivo* and *in vitro* constitutes a class of well-documentated reactions that take place readily because of electron availability on specific sites within the amine molecules (Smith 1965). Biological oxidation of a large number of classes of abiotic or xenobiotic foreign compounds has similarly been well-known to toxicologists and biochemists for a number of decades.

Oxidative metabolism of aromatic amines is now recognised as a prerequisite to their carcinogenic and genotoxic activity in animals, and ostensibly in man too. The noteworthy efforts of Miller and Miller and other workers has pointed towards hydroxylation of the amino nitrogen as being a crucial step along the carcinogenesis biopathway for these compounds. Biological N-hydroxylation was first recognized as a plausible metabolic reaction by Broide and Axelrod (1948). This enzymatic reaction was first demonstrated *in vitro* during studies on the mechanism of methemoglobin formation by aromatic amines (Kiese & Uehleke 1961). It has been identified by Miller and Miller (1966) as furnishing the initial step in the carcinogenesis pathway for aromatic amines. It typically involves a microsomally catalysed N-oxidation, as exemplified by the oxidation of 2-aminofluorene to N-hydroxy-2-aminofluorene. Alternatively, some aromatic amines may be first enzymatically N-acetylated and then N-hydroxylated to

give N-hydroxyarylamides, which are interconvertible to N-hydroxyarylamines through biological deacetylation and acetylation (Beland 1989). From the evidence available to date, apparently all carcinogenic aromatic amines must be converted to N-hydroxy compounds in order to exert their carcinogenic effects (Miller *et al* 1966), although all N-hydroxy amino compounds are not necessarily carcinogenic. This reaction of N-hydroxylation, together with subseguent esterification (*eg.* N-acetylation; see later), thus provides the key activation mechanism for converting the parent amines to reactive electrophiles *in vivo*. Here, the N-hydroxy metabolic derivative plays the part of the proximate carcinogen, which under appropriate conditions of further transformation and decomposition can yield the ultimate carcinogen species (see later).

N-hydroxy derivatives may in principle be derived either from the oxidation of a primary or secondary amine group, or from the reduction of a nitro, nitroso or N-oxide group. Insofar as carcinogens containing amino nitrogens are concerned, the former is the mechanism of relevance here. This oxidation may occur in a variety of organs in the mammalian body, but takes place primarily in the liver. The enzymes responsible for oxidation in the liver are contained within a network of intracellular membranes known as the endoplasmic reticulum, which contains an electron transport system collectively known as the mixed function

oxidase system (consisting of monooxygenases) that can metabolise a variety of drugs, carcinogens, steroids and so on. By using liver microsomal fractions, these conversions can be carried out *in vitro*, as shown by Kiese and Uehleke (1961). The microsomal fraction has to be supplemented with NADPH or a NADPH-generating system together with atmospheric oxygen.

It is readily apparent that there is considerable variation in the catalytic rates of *cytochromes P-450*. Experimental data suggest that the rate and extent to which an aromatic amine is N-hydroxylated depends both upon the identity of the *cytochromes P-450* present as well as upon the structure of the parent aromatic amine (Erturk *et al* 1967; 1970).

In general, the N-hydroxy derivatives are not usually the ultimate carcinogenic form of aromatic amines under physiological conditions, but are proximate forms that require further transformation along the carcinogenesis biopathway. A clue to these further metabolites was provided by Miller and and Miller (1969), whose work indicated that esters might provide a possible candidate for the ultimate form of carcinogenic aromatic amines. Since these early observations, a variety of N-esterified derivatives have been isolated and identified, which include the acetate, phosphate, sulphate and glucuronide forms. For the purpose of this dissertation, however, it was felt that the strong electrophilic reactivity of the arylnitrenium product or ester

ionisation furnished adequate ground for its identification as the most likely ultimate carcinogen. The ester metabolites would then be more properly classified as proximate carcinogens. The next paragraph summarises the opinion of certain workers regarding the likelihood of the ester itself acting as ultimate carcinogen.

The argument for the involvement of esters in the genotoxic biopathway runs as follows. If the N-hydroxy derivatives were invariably the ultimate form of the carcinogen, then they would be expected to react *in vitro* with cellular macromolecules to give derivatives similar to those found *in vivo* after administration of the parent compound. However, there are only a few reports in the literature of N-hydroxy derivatives binding to macromolecules *in vitro* (Marroquin *et al* 1970; Irving *et al* 1969). Under acidic conditions, this reaction can occur in certain cases (Kadlubar *et al* 1978; 1980) and this finding had led in part to the proposal that the acidity of urine may play a role in the bladder carcinogenicity of certain aromatic amines. Apparently, the esterified form possesses a greater intrinisic electrophilicity than the N-hydroxy derivative, or alternatively could easily give rise under an appropriate environment to yet another species of highly reactive electrophilic character (*viz.*, the arylnitrenium ion). The acidic condition of the bladder in dogs and in man could well provide the setting for dissociation of an N-esterified arylamine to yield the short-lived and unstable arylnitrenium ion

as the actual ultimate carcinogen. The following paragraphs discuss some N-esterified derivatives that have been identified.

ACETATE ESTERS: Esterification by acetate is a common conjugation mechanism in the metabolism of amines, sulfonamides and some aromatic amine acids. Bartsch *et al* (1972) reported an activation enzyme found in liver cell sap that acetylates hydroxamic acids. King *et al* (1979) isolated an N --> O acetyl transferase from the mammary tissue of rats. There is increasing evidence suggesting that enzymatic N-->O acyl transfer from arylhydroxamic acids may be an important pathway leading to formation of reactive carcinogenic speciess (King & Allaben 1980).

SULPHATE ESTERS: There is a good correlation between the activity of the N-sulphate ester metabolic system *in vivo* and the reactivity of N-hydroxy derivatives of arylamines *in vivo*, indicating that the sulphuric acid ester could be a major reactive ultimate carcinogen formed in the rat liver (DeBaun *et al* 1970). The sulphuric acid ester is very unstable, highly reactive and has a life in water of less than 1 minute (Jacoby *et al* 1980). Because of the short half-life and high reactivity, isolation of sulphate esters from the tissues or urine of animals exposed to aromatic amines has not been possible. As such, it would then seem unlikely that this species is directly involved in the bladder carcinomas so well-documentated in man an other mammals.

GLUCURONIDE ESTERS: The importance of glucuronidation as an activation mechanism for aromatic amines is under debate. The N-glucuronide of N-hydroxy 2-acetylaminofluorene (2AAF) reacts *in vitro* with nucleophiles although the reaction rate is much slower than with either N-acetoxy-2AAF or N-2AAF-N-sulfate (Marroquin & Coyote 1970). The N-glucuronides of N-hydroxy 2-naphthylamine, N-hydroxy 1-naphthylamine and N-hydroxy 4-aminobiphenyl at pH 5 were capable of conversion to reactive derivatives that could bind covalently to nucleic acids (Kadlubar *et al* 1977).

There are two competing metabolic pathways for primary arylamines, N-hydroxylation and N-acetylation. According to Lower's hypothesis (1979), N-acetylation is a detoxification pathway since it decreases the amount of aromatic amine available for N-hydroxylation. If the aromatic amine becomes N-hydroxylated, it can then be N-glucuronidated and transported to the bladder, where under acidic conditions the conjugate hydrolyses to release the putative ultimate carcinogen - the arylnitrenium ion, in which case N-glucuronidation would seem a possible activating pathway. What should be emphasized, however, is that having a fast acetylation phenotype does not always afford protection from the tumorigenic effects of aromatic amines.

DEACTIVATING METABOLIC PATHWAYS : Having presented the carcinogenically activating pathways that have been postulated and established, it now remains to dwell on some of the deactivating

metabolic pathways. Assuming the role of oxidase enzymes in this too, it would appear that, in general, oxidation and hydroxylation at the ring carbon atoms of aromatic amines would have a deactivating or detoxifying effect that detracts from the carcinogenic or genotoxic effects (Garner *et al* 1984). This has been attributed to the formation of phenolic compounds through C-hydroxylation, which are excreted from the host system in the form of soluble esters like glucuronides. The form of the esters also precludes the possibility of reactive electrophiles being generated in a potentially acidic medium here. In short, these observations may be summarised by contrasting the activating N-hydroxylation step with the deactivating C-hydroxylating one, the molecular aspects of which are studied in this chapter.

III.2 Activation of Some Important Aromatic Amines

It may be deemed noteworthy to review some of the current information on the metabolism of certain important aromatic amines, especially with regard to the carcinogenesis biopathway. Focus is laid on the activating pathways of N-hydroxylation and N-esterification. The following paragraphs delineate these findings for 2-napthylamine (2NA), 4-aminobiphenyl(4AB), benzidine (BZ), 2-acetylaminofluorene(2AAF), N-methylaminoazobenzene(MAB) :

2-Naphthylamine (2NA) : N-hydroxylation of 2-naphthylamine leads to a reactive product that is transported to the bladder as the N-glucuronide (Radomski & Brill 1970; Radomski *et al* 1973), which is itself unstable at low pH values (Kadlubar *et al* 1977). N-Glucuronides (Radomski 1970; Radomski *et al* 1973) of aromatic amines are thought to act as proximate bladder carcinogens through release of N-hydroxy compounds at the acidic pH of urine in humans and dogs (Kadlubar *et al* 1978). However, whether the N-hydroxide reacts directly with bladder tissue or requires further metabolism is not known.

4-Aminobiphenyl (4AB) : This compound was shown to be N-hydroxylated by rabbit and dog liver microsomes (Brill & Radomski 1971). N-hydroxy metabolites have also been identified in the urine of animals treated with parent amine (Boyland & Manson 1966; Brill & Radomski 1967). The hydroxylamine metabolites rather than the hydroxamic acid seems to be implicated in the activation process in bladder carcinogenesis (Radomski *et al* 1973). Acceptance of the hydroxylamine metabolite as being involved in this process was initially impeded by the fact that it is insoluble and unstable in aqueous media. This obstacle was removed, however, by the discovery of a glucuronide conjugate of N-hydroxy-4AB in the urine of dogs fed the parent amine. The conjugate was sensitive to acid pH but was stable at physiological pH; therefore, its transport from liver to bladder is possible.

Benzidine (BZ) : Benzidine has received relatively little attention in recent years, and, consequently, less is known about the metabolic activation of this compound. Martin and Ekers (1980) suggested that benzidine is activated by N-hydroxylation and that the synthesis of this unstable intermediate could be circumvented by synthesis of the more stable N-benzoyloxy ester. N-benzoyloxybenzidine itself proved too unstable for characterization, but the presumed compound was shown to react with DNA.

2-Acetylaminofluorene (2AAF) : Following N-hydroxylation, this compound can be activated in a variety of ways. In the presence of 3'-phosphoadenosyl- 5'-phosphosulphate and sulphotransferase enzymes, the highly reactive N-sulphate is formed (DeBaun *et al* 1970). Peroxidase enzymes can produce a nitroxide free radical (Floyd *et al* 1976) that dismutates with a second radical molecule to form N-acetoxy-2AAF and nitrosofluorene that can then be further metabolized. N,O-acyltransferase enzymes can transpose the acetyl group from the N-hydroxy-2AAF donor to an acceptor of the deacetylated N-hydroxy derivative of aminofluorene (2AF), to produce N-acetoxy-2AF. Esters can also be formed with the O atom of the N-hydroxy group leading, for example, to O-glucuronide formation *in vivo* in the presence of glucuronyltransferase (Hill and Irving 1967) enzymes. The most active of the metabolites produced *in vivo* is probably the N-sulphate (Kriek & Westra 1979).

N-Methylaminoazobenzene(MAB) : It has been demonstrated that N-hydroxy-MAB (Kadlubar *et al* 1976) was a substrate for microsomal sulfotransferase yielding a reactive species, the presumed MAB-N-sulfate (Kadlubar *et al* 1976), and it gave identical reaction products with nucleic acid to those obtained on administration of MAB to animals. Evidence has been presented that implicates the enzymatic production of a nitroxide free radical from N-hydroxy-MAB during the activation of MAB (Kimura *et al* 1979).

III.3 Methodology and Approach

III.3.1 Previous theoretical studies

Aromatic amine carcinogens and their metabolic derivatives have received some attention from theoretical chemists. The Huckel MO localisation energy of the carbon ortho to the amine group was used (Neely 1975) to serve as a screening criterion for a large set of aromatic amines, with some degree of success. Pack and Loew (1979) used the MNDO and MINDO/3 methods to study the enzymatic oxidation of amines with the help of a molecular model, and found that hydrogen abstraction, radical formation and N-oxide rearrangement were likely steps along the pathway.

Ford and Scribner (1981) performed theoretical calculations on nitrenium ions to elucidate the phenomenon of their electrophilic action. Semiempirical MO calculations at the MNDO level predict that the aryl and N-acetyl-N-arylnitrenium ions exist as ground state singlets resulting from the preferential stabilization of this state relative to the triplet (the ground state of NH^+ and the aliphatic nitrenium ions) by electron transfer from the phenyl substituent to the formally electron-deficient nitrogen atom.

III.3.2 Method and objectives

The wavefunctions for all molecular species studied in this chapter were calculated in the MNDO approximation (Dewar & Thiel 1977) using the MOPAC package (Stewart 1983). This methodology is, in fact, the method of choice for all the calculations embodied in this thesis. The reliability of the MNDO method has been established by systematic comparison with experimental data so that mean absolute errors are available for many ground state properties. The strength of MOPAC lies in its ability to provide accurate values of molecular properties from relatively fast calculations on medium to large molecules at about a fifth of the cost of *ab initio* methods. Geometries were optimized with no contraints for parent amines, N-hydroxylated forms, nitrenium ions

and N-, C- radicals of each amine by the *Davidon-Fletcher-Powell* algorithm (Fletcher & Powell 1963 ; Davidon 1968). Trial geometries were constructed from standard bond lengths and bond angles (Pople & Beveridge 1970).

The chief aims of the studies contained in this chapter may be outlined as follows :

(1) To make a assessment of the feasibility of N-hydroxylation of the parent amine as compared with C-hydroxylation, where the amino nitrogen and the most reactive carbon atom (the adjacent carbon atom to the amine group) in each species are taken into consideration. This is necessary in view of the observation that N-hydroxylation is activating while C-hydroxylation is deactivating.

(2) To calculate electrophilicities at the amino nitrogen and the adjacent carbon atom so as to arrive at a comparison of relative electrophilicity between the parent amine, the N-hydroxy derivative, and the arylnitrenium cation. This is done firstly to establish that the parent amines would be comparatively inert (as suggested by the ultimate carcinogen theory), as well as to attempt an identification of the actual ultimate carcinogen on the basis of reactivity and stability.

(3) To compare reactivity at the amine nitrogen and the adjacent carbon atoms as well as the thermodynamic stability for the various species along the metabolic route, in order to grade the various species with regard to electrophilic reactivity and thermodynamic stability. This significant for identifying which among the various species would be most likely to be the ultimate carcinogen and DNA modifier.

III.3.3 Theoretical indices used

A. *Indices for Amine N- and C- hydroxylations:*

The indices calculated from the MNDO-SCF wave-function to represent facility of parent amine hydroxylation are as follows :

(1) The frontier MO interaction terms T_l and T_h (*Eq III.1; Eq. III.2*) are contributors towards the net frontier MO interaction term T_{fo} (*Eq.III.3*) for estimating the extent of frontier MO interaction taking place between the amino hydrogen of the carcinogenand the triplet oxygen of the enzyme.

$$T_l = C_l^2 / (E_l - E_p) \qquad III.1$$

$$T_h = C_h^2 / (E_p - E_h) \qquad III.2$$

Here, C_h, C_l, E_h and E_l are the coefficients and energy levels of the HOMO and LUMO respectively (of the parent amines) which significantly involve the hydrogens, E_p being the energy level of the SOMO (singly occupied MO) of triplet oxygen. The triplet oxygen here is taken to represent the triplet oxygen contained in the heme moiety of the hydroxylating enzyme. The terms may be summed up as below (*Eq. III.3*) :

$$T_{fo} = T_h + T_l \qquad III.3$$

This term T_{fo} is actually a factor in the expression for the net frontier orbital interaction energy between amine and triplet oxygen as derived from second order perturbation theory (Klopman 1968).

(2) The Mulliken atomic charges on the relevant hydrogens in the parent amine (Q_{nh} and Q_{ch} for the hydrogens on the nitrogen and carbon respectively), which is significant in view of the nucleophilic nature of triplet oxygen

(3) The strength of the bond ruptured during the initial hydroxylation step (abstraction of a hydrogen atom), which may be measured by the Wiberg bond indices W_{nh} (for the N-H bond) and W_{ch} (for the C-H bond), where, for any bond between atoms A and B, *Eq. III.4* below holds good :

$$W_{ab} = \sum_{m}^{A} \sum_{n}^{B} P_{mn}^{2} \qquad III.4$$

The bond strength index is derived from the appropriate density matrix elements (Wiberg 1968).

(4) The stability of the radical obtained after abstraction of the hydrogen by the triplet oxygen, given in *Eq. III.5* below for the N-abstracted radical, and similarly for the C-abstracted one:

$$ArNH_2 + O^t \text{-----} > ArNH^{\cdot} + OH^{\cdot} + \Delta H_N \qquad III.5$$

B. *Indices for N- and C- atom electrophilicities:*

The following indices are chosen to represent electrophilicity towards DNA of the concerned atom for each species considered :

(1) The Mulliken atomic charges Q_N and Q_C (for the nitrogen and carbon respectively), in the parent amine, the N-hydroxy metabolite and the arylnitrenium ion, which is significant for the hard contribution to electrophilicity.

(2) The frontier interaction terms (T_N and T_C) for covalent attraction between the concerned atom of the amine or its

derivatives and the concerned atom of the base guanine, which may be expressed by *Eq. III.6* below :

$$T_x = \sum_{v}^{x} C_1^2 / (E_1 - E_h) \qquad III.6$$

where E_h is the energy of the highest occupied molecular orbital of guanine, while C_1 and E_1 are the relevant atomic orbital coefficients and energy of the lowest unoccupied molecular orbital of the relevant atoms on the carcinogenic species concerned. This frontier term T_x is actually a factor in the expression for the net frontier orbital interaction energy as derived from the total expression given by Klopman (1968). T_x is denoted as T_N and T_C for the nitrogen and the carbon respectively, which represents the soft contribution to electrophilicity.

C. *Thermodynamic stabilities of Arylnitrenium ion:*

Thermodynamic stability of *the* arylnitrenium ion relative to the parent amine or N-hydroxy metabolites are calculated here in terms of arylnitrenium ion generation from *(a)* the parent amine by ionisation following N-hydroxylation, *(b)* the parent amine itself without N-hydroxylation, *(c)* the N-hydroxy metabolite upon ionisation. Calculated enthalpies ΔH of the formal reaction processes mentioned above are shown in *Eq. III.7, Eq. III.8* and *Eq. III.9.*

$$ArNH_2 + O^t \text{ --------> } ArNH^+ + OH^- + \Delta H_o \qquad III.7$$

$$ArNH_2 \text{ ----------> } ArNH^+ + H^- + \Delta H_h \qquad III.8$$

$$ArNHOH + H_3O^+ \text{ ----------> } ArNH^+ + 2H_2O + \Delta H_s \qquad III.9$$

III.4 Hydroxylation of Parent Amines

Hydroxylation of 13 different parent amines at the amine nitrogen and at the adjacent carbon was studied by the MNDO SCF MO method using the indices mentioned in Sec. III.3.3 above, resorting to a triplet oxygen serving as the model for the heme moiety of the oxidase enzyme. The aromatic amines studied here include aniline (Anl), o-toluidine (oTol), m-toluidine (mTol), p-toluidine (pTol), 1-naphthylamine(1NA), 2-naphthylamine(2NA), 4-aminobiphenyl (4AB), benzidine(BZ), 2-aminofluorene(2AF), 2-acetylaminofluorene (2AAF), 3-aminofluorene (3AF), 3-acetylaminofluorene (3AAF) and N-methylaminoazobenzene (MAB). Table III.1 presents the MNDO calculated values for the indices concerning hydroxylation of parent amine by triplet oxygen (as given by *Eqns. III.3* and *III.4*), while Table III.2 presents the stabilities of the nitrogen and carbon amine radicals resulting from N- and C-hydrogen

Table III.1: MNDO calculated indices for N- and C-hydroxylation of parent aromatic amines using triplet oxygen model*

Amine	W_{nh}	$T_{fo}^{(nh)}$	Q_{nh}	W_{ch}	$T_{fo}^{(ch)}$	Q_{ch}
Anl	0.950	0.88	0.113	0.964	0.20	0.060
oTol	0.955	0.91	0.114	0.963	0.98	0.059
mTol	0.959	1.12	0.113	0.963	1.68	0.064
pTol	0.959	0.97	0.115	0.963	1.04	0.063
1NA	0.955	0.54	0.111	0.963	0.37	0.060
2NA	0.956	0.52	0.114	0.963	0.48	0.061
4AB	0.958	0.82	0.115	0.962	0.76	0.064
BZ	0.959	0.54	0.114	0.963	0.44	0.063
2AF	0.959	1.32	0.115	0.963	1.02	0.062
2AAF	0.936	1.31	0.149	0.962	1.02	0.103
3AF	0.959	1.27	0.114	0.963	1.09	0.062
3AAF	0.936	1.29	0.149	0.962	1.04	0.103
MAB	0.957	0.98	0.119	0.963	1.01	0.066

*All indices in atomic units

abstractions by triplet oxygen (see *Eq. III.5*). The structures of some of the aromatic amines studied have been depicted in Fig. III.2.

In general, the calculated data here indicates that, in the competition between N- and C-hydroxylation pathways in the parent amines, the former is predicted to be definitely favoured. The strength of the bond ruptured during initial hydrogen abstraction is invariably found to be stronger for the C-H case (W_{ch}) than the N-H case (W_{nh}), where the Wiberg bond index has the value range from 0.936 to 0.959 for the N-H bonds, but the larger range from about 0.962 to 0.963 for the C-H bonds. This is also indicated by the uniform larger positive charge on the amine hydrogen (Q_{nh}) than the carbon hydrogen (Q_{ch}), assuming the nucleophilic character of the triplet oxygen. The T_{fo} term for covalent interaction is also generally, but not always, larger for N-hydrogen abstraction than C-hydrogen abstraction. The most striking indication is given by the invariable greater stability of the N-hydrogen abstracted amine radical (ΔH_N) over the C-hydrogen abstracted radical (ΔH_C), as seen from the data of Table III.2. The radical stabilities are in the range of -39 to -52 kcal/mol for the N-hydrogen abstractions, while they are about only -10 to -23 kcal/mol for the C-hydrogen abstractions.

The inference that N-hydroxylation would predominate over C-hydroxylation would speak of the relative abundance of

Table III.2: Enthalpies for production of N and C radicals by hydrogen abstraction from parent aromatic amines*

Amine	ΔH_N	ΔH_C
An1	-39.1	-11.9
oTol	-40.6	-14.3
mTol	-39.5	-15.2
pTol	-39.5	-13.6
1NA	-47.1	-20.2
2NA	-45.3	-10.4
4AB	-42.2	-16.3
BZ	-42.5	-16.5
2AF	-45.5	-19.2
2AAF	-52.4	-22.8
3AF	-42.0	-18.9
3AAF	-51.2	-21.9
MAB	-45.4	-19.2

*All values in kcal/mole

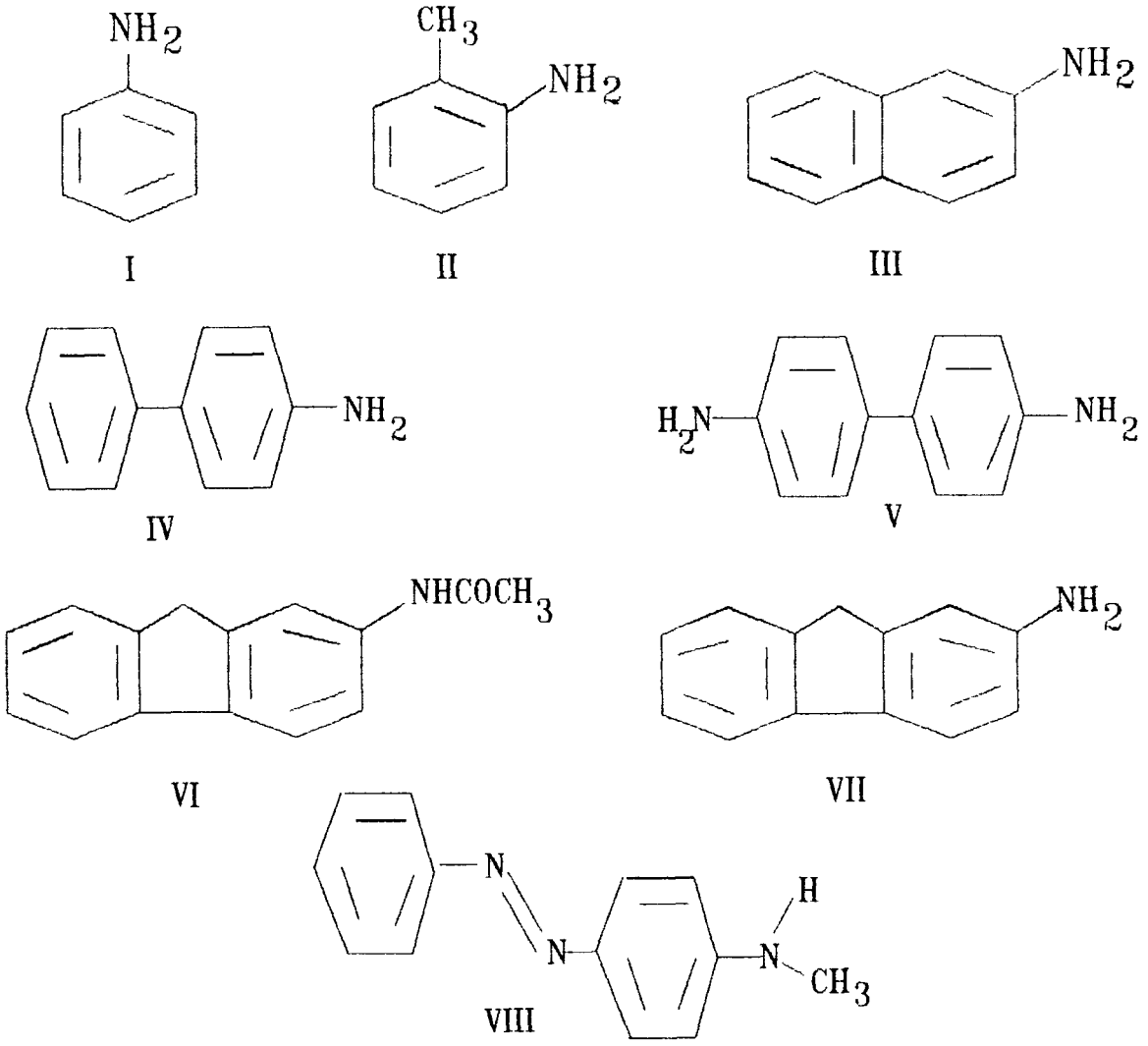


Fig.III.2: The structures of some of the aromatic amines studied. I: aniline(Anl); II: *o*-toluidine(*o*Tol); III: 2-naphthylamine(2NA); IV: 4-aminobiphenyl (4AB); V: benzidine(BZ), VI: 2-acetylaminofluorene (2AAF); VII: 2-aminofluorene(2AF); and VIII: N-methylaminoazobenzene (MAB).

N-oxidised products (activating) over C-oxidised products. This may be offset only by the larger number of ring carbon atoms present in the parent amine in comparison to the single amino nitrogen present. Both these effects may be expected to contribute in determining the balance of activating versus deactivating pathways, which in turn would be expected to also determine the net genotoxic or carcinogenic effect. The complex interplay between all these effects is currently still beyond the scope of this present work, which is why prediction and rationalisation of relative carcinogenic potencies among a group of aromatic amines is not attempted here.

III.5 Proximate and Ultimate Carcinogenic Species

Electrophilic reactivity of the ultimate carcinogen is a feature of the original theory, and is expected to characterise the actual ultimate carcinogen species here. Comparison of N- and C-atom electrophilicities among the various species along the carcinogenesis pathway is called for owing to the following reasons :

(1) As per the criteria of the theory for indirect-acting carcinogens, electrophilic reactivity may be expected to be minimal for the parent carcinogen, and optimal for the ultimate

carcinogen species. Calculated indices for electrophilicity among the various species along the way would help to justify the need for chemical transformation of the parent species owing to their expected inertness.

(2) Furthermore, the level of electrophilicity as gauged from the value of these indices would also help to select the actual candidate most likely to fulfil the role of the real ultimate carcinogen among a series of candidates (here the N-hydroxylamine, the arylnitrenium ion and the esterified amine).

(3) Both N- and C-atom electrophilicities are considered since the structures of the adducts formed between carcinogen and DNA constituents indicate that both the amino nitrogen as well as the adjacent carbon on the ring are involved in covalent bonding between the carcinogen moiety and the DNA base.

Tables III.3 and III.4 present the MNDO calculated values of the electrophilic reactivity indices (see Sec. III.3.3 above) for the amine nitrogen and the most reactive carbon on the ring, these being given here for the 13 different aromatic amines considered in Sec. III.4 above, comparisons being made between the parent amine, the proximate carcinogen (the N-hydroxylamine) and the putative ultimate carcinogen (arylnitrenium) species.

These results in general predict that the electrophilicity of the putative ultimate carcinogen species (the arylnitrenium ion) would be much greater than that for the other two species. This

Table III.3: Comparison of N-atom electrophilicities of Parent, Proximate and Ultimate Carcinogens for Different Aromatic Amines*

Amine	$ArNH_2$		ArNHOH		$ArNH^+$	
	Q_N	T_N	Q_N	T_N	Q_N	T_N
An1	-0.221	0.64	-0.107	0.64	-0.065	1.49
oTol	-0.229	0.63	-0.114	0.67	-0.018	1.44
mTol	-0.228	0.59	-0.110	0.64	0.002	1.42
pTol	-0.229	0.65	-0.109	0.65	-0.006	1.40
1NA	-0.230	0.26	-0.114	0.63	-0.074	1.45
2NA	-0.229	0.28	-0.110	0.35	-0.033	1.90
4AB	-0.230	0.31	-0.078	0.71	-0.046	1.64
BZ	-0.229	0.19	-0.108	0.65	-0.090	0.79
2AF	-0.230	0.21	-0.078	0.53	-0.063	1.26
2AAF	-0.336	0.16	-0.221	0.58	-0.164	2.31
3AF	-0.229	0.19	-0.080	0.39	-0.024	2.51
3AAF	-0.333	0.19	-0.232	0.44	-0.160	2.39
MAB	-0.231	0.56	-0.110	0.58	-0.051	1.08

*All values in atomic units

Table III.4: Comparison of C-atom Electrophilicities for Parent, Proximate and Ultimate Carcinogens of Different Aromatic Amines*

Amine	$ArNH_2$		ArNHOH		$ArNH^+$	
	Q_c	T_c	Q_c	T_c	Q_c	T_c
Anl	-0.091	0.75	-0.068	0.75	0.183	6.63
oTol	-0.103	0.69	-0.049	0.84	0.088	1.49
mTol	-0.086	0.69	-0.031	0.68	0.214	1.54
pTol	-0.098	0.61	-0.059	0.58	0.200	1.53
1NA	-0.107	0.31	-0.048	0.36	0.198	2.78
2NA	-0.089	0.45	-0.066	0.45	0.188	4.23
4AB	-0.098	0.60	-0.046	0.48	0.153	1.40
BZ	-0.099	0.43	-0.060	0.48	0.103	1.40
2AF	-0.043	0.54	-0.034	0.54	0.153	1.14
2AAF	-0.088	0.59	-0.022	0.57	0.161	1.34
3AF	-0.097	0.57	-0.073	0.49	0.207	3.57
3AAF	-0.087	0.66	-0.066	0.52	0.189	3.78
MAB	-0.105	0.65	-0.064	0.72	0.149	1.33

*All values in atomic units

disparity in reactivity is particularly noteworthy between the parent amine and the arylnitrenium ion species. The N-hydroxylamine species occupies an intermediary position along the scale of electrophilic reactivity, where the general order for electrophilic reactivity predicted by the two indices is as follows : $ArNH_2$ < ArNHOH < $ArNH^+$. These inferences remain the same regardless of whether N-atom or C-atom reactivity is taken into account (Fig. III.3, Fig. III.4). As such, the predicted inertness of the parent amines justifies their role as indirect-acting carcinogens which require chemical conversion to a more reactive species *in vivo*, as per the concepts of the ultimate carcinogen theory. The intermediary reactivity of the N-hydroxylamine species perhaps would warrant their description and designation as "proximate carcinogens" rather than ultimate carcinogens. As a matter of fact, reactivity of the N-hydroxylamine seems only slightly greater than the parent amine, further justifying the description here as a proximate carcinogen.

Calculations were also performed for deducing N- and C- atom electrophilicity in the acetate esters of 2NA and 2AAF. The Q_N, T_N, Q_C, and T_C terms for the 2NA case predict greater N-atom and C-atom electrophilicity for the ester over the N-hydroxylamine. The index values for the ester are -0.093 (Q_N), 0.577 (T_N), -0.015 (Q_C) and 0.585 (T_C). The corresponding terms for the 2AAF ester are -0.175 (Q_N), 0.555 (T_N), -0.041 (Q_C) and 0.654 (T_C),

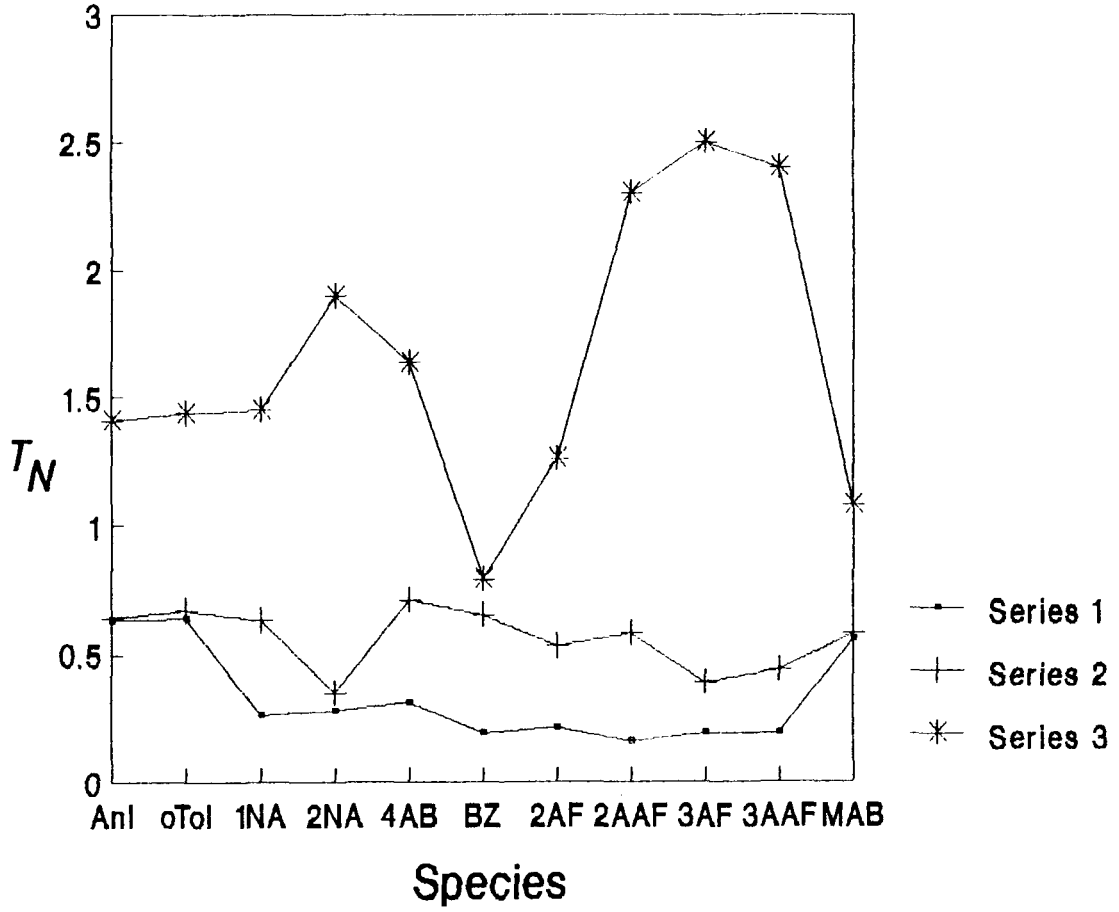


Fig.III.3: Comparison of N-atom electrophilicity (T_N) among the various species. Series 1: parent amines; Series 2: N-hydroxylamines; Series 3: arylnitrenium ions.

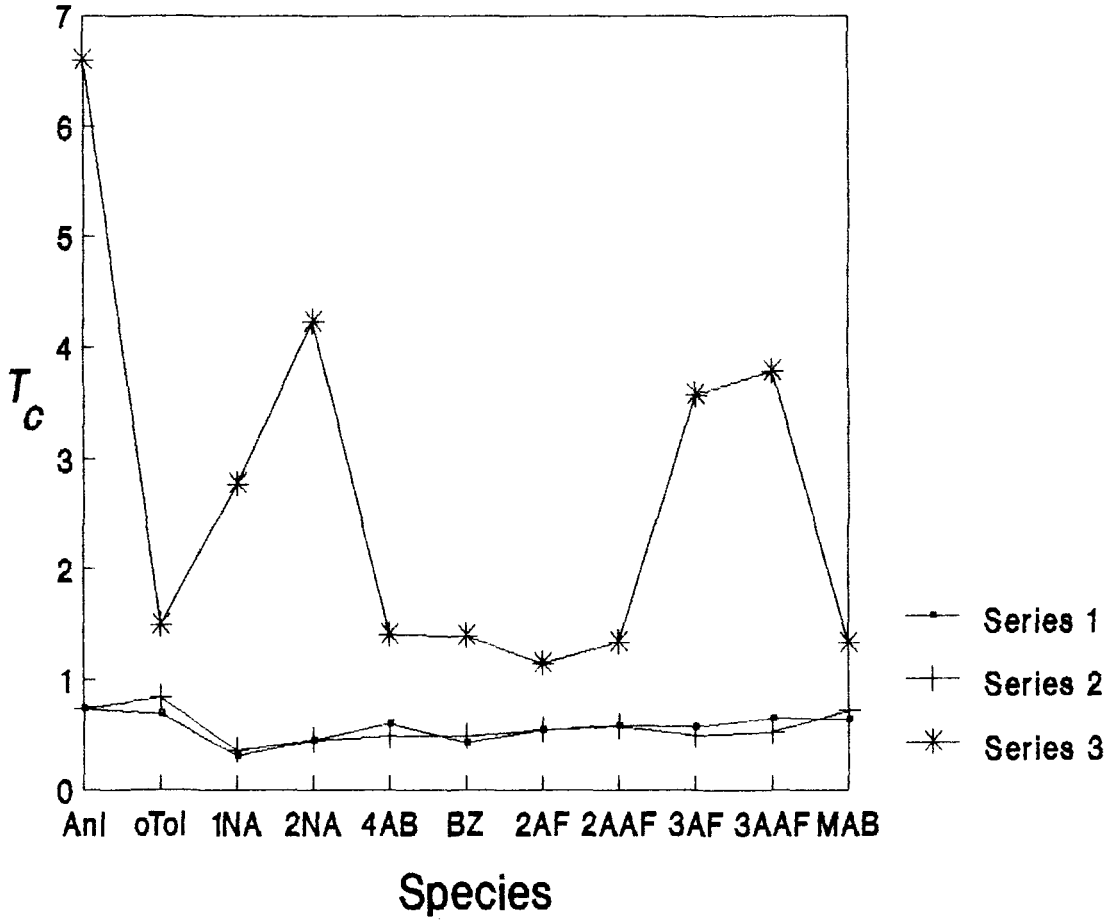


Fig.III.4: Comparison of C-atom electrophilicities (T_C) among the various species. Series 1: parent amines; Series 2: N-hydroxylamines; Series 3: arylnitrenium ions.

indicating a fairly close comparison in N- and C-atom electrophilicities between the ester and the corresponding N-hydroxylamine.

Only the arylnitrenium ion species appears to really merit the appellation of "*ultimate carcinogen*", where its reactivity is along the order of other putative ultimate carcinogens like the alkanediazonium ion species for N-nitroso carcinogens, as judged from a comparison of values of the electrophilicity indices (Lyngdoh, unpublished work).

The electrophilicity was studied in two sites, *viz.* at the amine nitrogen and at the carbon adjacent to the amino group site (see Tables III.3 and III.4 for the parent, proximate and ultimate carcinogen species). For all three species, it is predicted from a comparison of values of the T_{fo} terms that the carbon site would have a greater covalent term than the nitrogen site. However, the atomic charges predict a greater positive charge for the carbon over the nitrogen. This is best borne out by the index values for the nitrenium ion species, which on all accounts (both electrostatic and covalent) would be the strongest electrophile among the three different species, regardless of whether the nitrogen or carbon atoms are considered. Since the two aspects of charge-controlled and frontier-controlled aspects of reactivity thus seem to oppose each other, it may be inferred that both these sites are neither very hard nor very soft as electrophiles, but

possess an intermediary status. It will be seen in the next Chapter that these findings have a bearing upon the product distribution of carcinogen-DNA base adducts.

The quantities ΔH_o, ΔH_h and ΔH_s of Table III.5 represent the calculated enthalpies of the formal reaction processes corresponding to various routes for generation of the arylnitrenium ion, as given by the *Eqs. III.7, III.8* and *III.9*. The enzymatic hydroxylation route for generation of the ultimate carcinogen (ΔH_o) was compared with the alternative transformation route of hydride ion loss (ΔH_h), and the former was found to be definitely the more favoured one energetically. This points to the energetic advantage of biological oxidation over that of heterolytic hydride ion loss. The gas phase ionic dissociation of the N-hydroxylamine, as given by the ΔH_s index, is predicted to be thermodynamically facile, having the range from -25 to -40 kcal/mole. The corresponding enthalpies of ionic dissociation of the acetate esters of 2NA (-35.7 kcal/mole) and 2AAF (-34.1 kcal/mole) do not indicate any marked difference in arylnitrenium ion generation facility between the ester and the N-hydroxyl amine, at least from the thermodynamic perspective.

The various indices of this Chapter fail to reveal any clear demarcation between active and inactive members of the class, so these simple chemical criteria cannot be used to screen well for the presence or absence of carcinogenicity.

Table III.5: Enthalpies of formal reaction processes (Eqs.III. 7, 8, 9) for different pathways of arylnitrenium ion formation*

Amine	ΔH_o	ΔH_h	ΔH_s
An1	154.4	293.6	-25.1
oTol	150.9	290.1	-27.5
mTol	153.6	292.7	-25.6
pTol	151.8	290.9	-27.7
1NA	138.6	277.7	-40.4
2NA	145.4	276.7	-34.5
4AB	145.0	284.1	-37.4
BZ	137.1	276.2	-42.8
2AF	138.3	277.5	-45.9
2AAF	143.4	282.2	-35.3
3AF	147.3	286.4	-37.0
3AAF	142.5	281.6	-32.2
MAB	143.6	282.8	-35.9

*All values in kcal/mole

The indices used here prove coherently capable of identifying the most likely species that functions as the actual ultimate carcinogen among the various candidate species considered, which choice is fulfilled in identification of the arylnitrenium ion as the real ultimate carcinogen and DNA modifier.

III.6 Conclusions

The calculations of this Chapter show that the activating N-hydroxylation route would be more facile than the deactivating C-hydroxylation route, which indicates one mechanistic aspect proving favourable for the genotoxicity of aromatic amines upon enzyme-mediated metabolism.

The results also indicate a marked difference in electrophilicity among the various species along the carcinogenesis route. On the basis of reactivity criteria for single atoms and bonds obtained from molecular orbital calculations, it has been shown that the arylnitrenium ion metabolites are definitely much more *electrophilic* than their parent or proximate counterparts and also exhibit this electrophilicity at two sites. The relative inertness of the parent amine as thus predicted here hence calls for the necessity of its conversion to a more electrophilic species *in vivo* if DNA modification is to be effected, which role is played by the arylnitrenium ion as *ultimate carcinogen*.

Chapter IV

CHAPTER IV

ADDUCT FORMATION BETWEEN DNA BASES AND ARYLNITRENIUM IONS

IV.1 Interaction of Aromatic Amines with DNA

IV.1.1 DNA modification by xenobiotic agents

The possibility of bonded interactions of chemical carcinogens with cellular macromolecules was first noted with findings that aminoazo dyes and benzo[a]pyrene are bound to proteins in tissues in which they produce tumours (Freeman 1980; Beland *et al* 1981). Covalent binding between chemical carcinogens and both nucleic acids and proteins in tumour-susceptible tissues was in time found to be quite a general phenomenon, which has been the subject of many reviews (Miller & Miller 1981). Although the extent of binding to DNA and RNA, but not to proteins, generally showed reasonable correlation with the carcinogenic potency, this was not always true. Furthermore, though the vast majority of adequately

studied carcinogens bind covalently to cellular macromolecules *in vivo*, some exceptions have been noted so far. One is the carcinogenic and mutagenic antitumour agent *adriamycin* which binds *in vivo* to DNA in a non-covalent fasion (Singer & Grunberger 1983).

After examination of the binding of a large number of chemical carcinogens to nucleic acids, E.C. and J.A. Miller proposed the ultimate carcinogen theory (reviewed in Sec. I.3) which proposes that the actual cancer-inducing agents in chemical carcinogenesis are highly reactive electrophiles that form covalent linkages with nucleophilic residues in cellular macromolecules. Carcinogens that are not electrophiles as such when administered to the host undergo metabolism by a mixed function oxidase system to reactive ultimate carcinogenic forms. Since the formation of nucleic acid-carcinogen adducts is likely to be the key factor at least in the initiation stage of carcinogenesis, the elucidation of the chemical structures and conformations of these adducts is of much importance here. It may be worthwhile to briefly note the general experimental approaches used to obtain information on the types of carcinogen-DNA adducts formed in cells, tissues and hosts exposed to chemical carcinogens.

The approach used to isolate carcinogen-DNA adducts from animal hosts involves the initial exposure to the carcinogen (single-dose, multiple-dose or chronic exposure), followed by

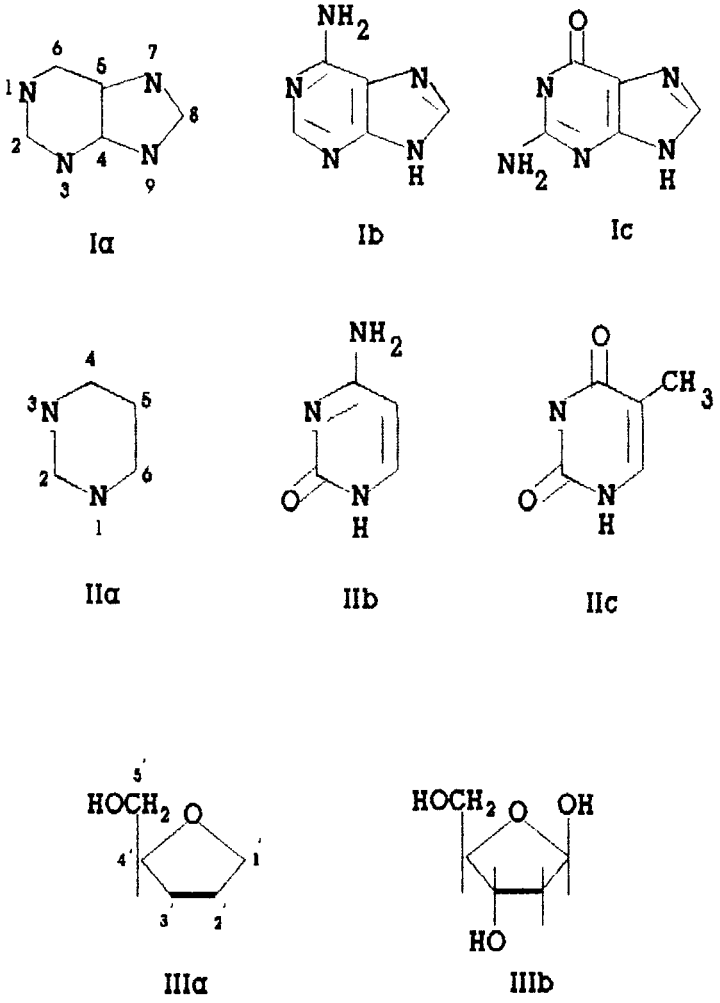


Fig.IV.1: Structures and numbering systems for DNA constituents. Ia: atomic numbering for purines; Ib: adenine (A); Ic: guanine (G), IIa: atomic numbering for pyrimidines; IIb: cytosine (C); IIc: thymine (T); IIIa: atomic numbering for sugar; IIIb: β-2'-deoxyribose (in DNA).

sacrifice of the animal from which the concerned tissues are extracted (which may or may not be neoplastically transformed). The process of DNA separation then commences, which may be done by submitting the relevant cell mass to ultracentrifugation. The separated and purified DNA is then hydrolysed and the nucleoside components separated out by techniques like chromatography (HPLC). The isolation and identification of the particular modified nucleosides may be performed by a variety of methods, including use of monoclonal antibodies, radioimmunoassay and the standard methods of molecular spectroscopy for structure determination, including NMR, circular dichroism and mass spectroscopy.

Formation of carcinogen-DNA adducts may also be carried out *in vitro* by exposure of tissue cultures or cell lines or even naked DNA to the carcinogen. *Indirect-acting* carcinogens would of course require the appropriate metabolising environment in the form of microsomal fractions or purified enzymes.

IV.1.2 DNA modification by aromatic amine carcinogens

One of the best studied interactions of carcinogens with macromolecules is that of covalent attachment of the reactive metabolites of aromatic amines (*arylnitrenium ions*) to different sites of DNA bases (Westra *et al* 1976). These various sites are conventionally numbered for the DNA bases in Fig. IV.1. The

following paragraphs give some specific information on the chemical structure of the products of interaction between DNA components and certain well-studied aromatic amines :

(1) **Acetylaminofluorene** : Early in the 1960's, with the recognition of proximate and ultimate carcinogenic forms of carcinogens by the Millers, the carcinogen acetylaminofluorene (AAF) was used to study its nonenzymatic reaction with nucleic acids. The major products formed in these reactions were characterized and shown to be identical with covalent adducts formed *in vivo* when the parent or proximate carcinogen N-hydroxyacetylaminofluorene (N-OH-AAF) was administered to rats (Howard *et al* 1981). Three types of DNA adducts were determined by using *Sephadex* LH-20 column chromatography in combination with HPLC (Howard 1981). It was shown that the major product (80%) obtained from hydrolysates of the modified DNA is N-(deoxyguanosin-8-yl)-AAF (Krick 1969; Irving & Veazey 1969). The remaining fraction of the AAF residues (20 %) was identified as 3-(deoxyguanosin-N-yl)-AAF (Westra *et al* 1976). After administration of AAF to rats *in vivo*, a third DNA adduct was also detected. It represented a deacetylated form, *viz.* N-deoxyguanosin-8-yl)-acetylaminofluorene (Krick & Westra 1980).

(2) **2-Naphthylamine** : 2-Naphthylamine (2NA) is metabolically activated to N-hydroxy-2-naphthylamine (N-OH-2NA), which is converted under the slightly acidic conditions of urine to an

electrophilic arylnitrenium ion capable of binding covalently to cellular macromolecules (Kadlubar *et al* 1977). When Kadlubar and coworkers reacted N-OH-2NA with DNA *in vitro* at pH 5.0 and hydrolyzed the DNA, they obtained three nucleoside-arylamine adducts, which were isolated and identified by chemical and spectrometric analyses. The two major ones involve binding to guanine, where the purine ring opened derivatives of N-(deoxyguanosin-8-yl)-2NA and 1-(deoxyguanosin-N-yl)-2NA were characterised. The third adduct is 1-(deoxyadenosin-N-yl)-2NA with adenine (Kadlubar *et al* 1980).

(3) **Benzidine** : In the first stage, benzidine (BZ) may be N-acetylated to yield acetylbenzidine (ABZ), which in turn is N-hydroxylated on the primary amine fuction to yield a proximate carcinogen N-hydroxy-N'-acetylbenzidine (N-OH-N'-ABZ). The chemical identity of the DNA adduct formed *in vivo* was identified by NMR and mass spectra as N-(deoxyguanosin-8-yl)-N-ABZ (Martin *et al* 1982).

(4) **Aminobiphenyl** : This apparently acts *via* conversion to the ester form. The sulphate ester of N-hydroxy-4-aminobiphenyl-acetamide reacts with DNA *in vitro* to give C^8- and N^2-deoxyguanosine adducts. Level of binding for this ester is considerably lower than those seen with the equivalent AAF ester (Krick & Westra 1978; 1979).

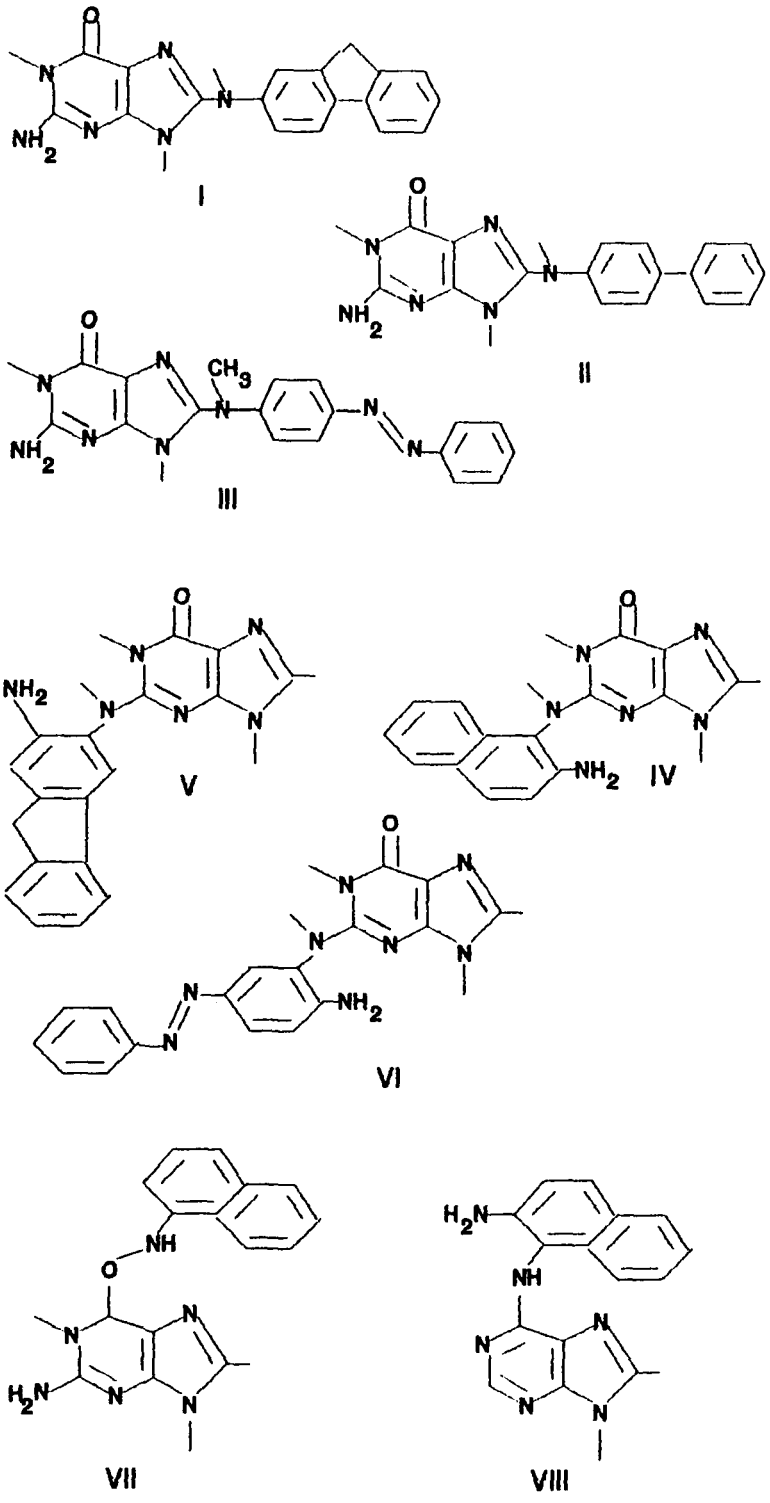


Fig.IV.2: Some of the adducts of the aromatic amines with different DNA base sites. I: N-2(8-deoxyguanosinyl)AF; II: N-4 (8-deoxyguanosinyl) AB; III: N-methyl-N-(8-deoxyguanosinyl)-4-phenylazoaniline; IV: 1-(N-deoxyguanosinyl) -2NA; V: 3-(N-deoxyguanosinyl)-2AF; VI: 1-(N-deoxyguanosinyl)-MAB; VII: N- (O-deoxyguanosinyl)-1NA; VIII: 1- (N-deoxyadenosinyl)-2NA.

In general, it may be summarised that the major products of DNA interaction with carcinogenic aromatic amines have been largely identified as the N-(deoxyguanosin-8-yl) adducts. The less abundantly formed products include those adducts where the ring carbon adjacent to the nitrogen of the arylnitrenium ion is covalently bonded at the N^2-guanine position (Kriek & Westra 1979). There is also evidence for adducts where the reactive carbon as well as the nitrogen of the arylnitrenium ion is bound to the O^6-guanine site (Kadlubar *et al* 1981).

Some of the above-mentioned adducts are portrayed in Fig. IV.2 in their neutral (deprotonated) form.

IV.2 Mechanisms of Adduct Formation with DNA bases

In consonance with the ultimate carcinogen theory, aromatic amines attack nucleic acids after prior metabolism to the arylnitrenium cation species. As seen from the preceding Section, it is evident that the arylnitrenium ion can utilise two of its atoms for electrophilic attack, *viz.* the imino nitrogen and the carbon closest to this nitrogen which is in conjugation with it. Current opinion adheres to the conclusion that, out of the various components of DNA, only the DNA base moieties upon modification are relevant for mutagenesis and cancer. The sites for attack on DNA bases include the N^2-atom of guanine, the C^8-atom of guanine

or adenine, and occasionally the O^6-atom of guanine. It appears that the amino nitrogen of the nitrenium ion never bonds to another nitrogen on the DNA bases; neither does the adjacent carbon of the ion ever bind to carbon atoms of DNA bases. Rather, the imino nitrogen bonds to the C^8-atom of purines, while the adjacent carbon on the cation binds invariably to an exocyclic nitrogen of the DNA bases (like the N^2-atom of guanine). This observation calls for an explanation, here in terms of molecular orbital indices for the strength of interaction.

Mechanistically, the reaction between ultimate carcinogen and DNA bases may be classified as being of the **electrophilic substitution** type, where the electrophile displaces a proton on the nucleophile. This happens in two ways. For the former, the incoming electrophilic nitrogen of the cation pushes out a proton from the nucleophilic carbon site of attack on the bases; this is true for interaction between the imino nitrogen of the ion and the mildly nucleophilic C^8-carbon on purine bases. For the second case, the incoming electrophilic carbon on the carcinogenic ion pushes out a proton from the nucleophilic nitrogen of the base; this is true for the interaction between the adjacent carbon of the nitrenium ion and the amine nitrogens of purines.

Theoretical rationalisations for the product distribution of carcinogen-DNA adducts have to tackle the observation that adducts involving C-C bonding or N-N bonding never occur. The

arylnitrenium nitrogen may attack only a carbon on a DNA base; similarly the arylnitrenium electrophilic carbon may attack only an amino nitrogen on the DNA base. Hard-soft acid-base concepts may be fruitfully exploited to furnish predictions and explanations regarding the product distribution. Furthermore, the enthalpy of adduct formation may be invoked to explain the relative abundances of the C^8-G and N^2-G products.

IV.3 Methodology and Approach

IV.3.1 Previous Theoretical Studies

A fair amount of theoretical work has been done by various groups on aromatic amines and their interaction with DNA bases, which is described briefly in the following paragraphs :

The binding of 1- and 2-naphthylnitrenium ions to DNA bases and a dinucleotide was studied by Gresh (1982) using *ab initio* wave functions, which served to explain some observed differences in DNA modification by 1- and 2-naphthylamine.

Loew *et al* (1979) used semiempirical MO methods to study the activation of aromatic amines to their electrophilic derivatives and the binding of these to DNA bases. The relevant theoretical quantities were invoked in an attempt to correlate with mutagenic

and carcinogen potency indices and also to rationalise the specificity of adduct formation with DNA observed experimentally.

Minimized potential energy calculations on the three-dimensional structures of carcinogen-modified DNA were carried out on a large number of systems, using a variety of models (reviewed by Broyde & Hingerty 1985).

Minimized conformational potential energy calculations have been performed on the major adduct of N-acetyl-2-aminofluorene (2AAF) and 2-aminofluorene (2AF) with dCpdG. The DNA backbone conformations that produce base displacement, about which no information was previously available, were ascertained (Hingerty & Broyde 1982).

Energy minimized structures of carcinogen-DNA adducts for 4-aminobiphenyl have been reported. Conformations pertinent to the A and B helical conformation adopted by DNA of random sequence and the conformational effect of 4-aminobiphenyl on DNA were reported (Broyde & Hingerty 1986).

Energy minimized structures of DNA modified by the aromatic amines 2-acetylaminofluorene and 2-aminofluorene, for which no experimental atomic resolution data exist, were presented (Hingerty & Broyde 1986). These have been computed with a new molecular mechanics program specifically designed to define distortions imposed by such adducts, and employing a rational

strategy for searching the conformation space of a DNA molecule with covalently linked carcinogens.

Minimized potential energy calculations were carried out for visualization of an AAF induced frameshift mutation in B-DNA (base displacement) (Broyde & Hingerty 1987). Model building by computer graphics was employed here.

Minimized potential energy calculations were employed to locate and evaluate energetically a number of different models for DNA modified at C^8- of guanine by AAF. Three different duplex nonamer sequences were investigated. Two types of structures were found in which guanine remains *syn* and the AAF is placed in the minor groove of a B-DNA helix (Shapiro *et al* 1989).

Unusual hydrogen bonding patterns in 2AF and 2AAF modified DNA were reported by Broyde *et al* (1990). A duplex 11-mer was investigated in which a central G was modified at the C^8-atom. High resolution NMR data were employed to search for unconstrained energy minimized structures consistent with it.

Minimized conformational potential energy calculations were performed for N-2-acetylaminofluorene (AAF) linked to dCpdG at the guanine amino group. This is a model for the minor AAF adduct observed in DNA, whose conformational influence has been difficult to asscertain. A global minimum energy conformation was computed with torsional angles like those of the dCpdG residue of Z-DNA. This conformation was incorporated into a large polymer model at a

B-Z junction, with the carcinogen residing in the groove in the Z direction (Hingerty & Broyde 1983).

A review on predicting structures of DNA and carcinogen-modified DNA by build-up techniques was published (Hingerty *et al* 1991).

Molecular modelling in conjunction with molecular mechanical calculations (AMBER) was used for bulky (three-dimensional) adducts with DNA to assess if the carcinogen moiety of the adduct might be able to shift the position of the base moiety in such a way that misreplication might be facilitated. A variety of mispairing events might result from adduct induced base-rotation (Loechler 1989).

IV.3.2 Aims and Objectives

This chapter focusses on DNA modification by the ultimate carcinogenic forms of 2-naphthylamine (2NA), 4-aminobiphenyl (4AB), benzidine (BZ), 2-acetylaminofluorene (AAF), 2-aminofluorene (2AF), and N-methylaminoazobenzene (MAB), all of which have well-established carcinogenicity (Garner *et al* 1984). The following constitutes the aim and objective of this study :

To provide rationalisations and theoretical predictions of the product distribution of DNA-carcinogen adduct formation through use of a semiempirical SCF-MO model (the MNDO SCF-MO method).

IV.3.3 Theoretical Procedure

The theoretical indices used here were derived :

1. in terms of the isolated reactants,
2. in terms of the carcinogen-DNA interaction energies at nonbonding internuclear distances
3. in terms of the enthalpy of the adduct-formation reaction.

1. *Indices involving isolated reactants:* Such indices represent only the individual tendency of each separated reactant to favour the reaction. Here, these were calculated for both the arylnitrenium ion as well as the DNA base, and include :

(a) The Mulliken charge on the relevant atoms (Q_n or Q_c, for the nitrenium ion and Q_b for the base) which may be expressed as single point charges or else as the net charge (Q_{nh} or Q_{ch}) on the concerned atom of the nitrenium ion including attached hydrogens (as this collective charge would make itself felt keenly upon the nucleophilic sites by reason of proximity). This quantity reveals the electrostatic (hard) component of the interactions.

(b) The energy of the highest occupied molecular orbital (E_h) of the DNA base, and the energy of the lowest unoccupied molecular orbital (E_l) of the nitrenium ion, in which the relevant atoms participate significantly through the sizable coefficients of their atomic orbitals. These are of bearing for the covalent (soft) or frontier-controlled aspects of the interactions.

(c) The frontier electron density Q_{fo} on the concerned atom, which is given by *Eqn. IV.1* below :

$$Q_{fo} = c_s^2 + c_{px}^2 + c_{py}^2 + c_{pz}^2 \qquad IV.1$$

the C terms being the coefficients of the atomic orbitals. This quantity Q_{fo} reflects upon the soft contribution to carcinogen-DNA interaction.

(d) The molecular electrostatic potential minima associated with the DNA base sites which Pullman and Pullman (1980) have computed for various sites in B-DNA, where the order is : C^8-G > N^2-G. The NH_2 group is generally considered as a secondary site for hard electrophilic attacks, where the energy minima associated with the isolated base guanine corresponds to a repulsion.

(e) The steric accessibilities associated with the DNA base sites : Using a water molecule probe, steric accessibilities at various sites in double-helical B-DNA have been calculated by Lavery *et al* (1981), the order of magnitude being again C^8-G > N^2-G.

2. *Indices in terms of interaction energy:* The interaction energy between nitrenium ions and DNA base sites at non-covalently bonded internuclear distance may be taken as representative of the affinity between the two species, and may be drawn upon to rationalise or predict carcinogen-DNA reactivity. This is considered in the background of the electrophilic attack of nitrenium ions to DNA bases, which is treated here by invoking

Pearson's principle of hard and soft acids and bases (1966). The approach used here to calculate non-bonded affinities between the electrophile and the nucleophile stems from the approach of Klopman (1968). The concepts of charge- and frontier-controlled aspects of reactivity were utilised as below.

In simplified form, the energy of interaction E_{ab} between an electrophile A and a nucleophile B may be expressed as a sum of *an electrostatic term* E_{es} *and a covalent term* E_{cv} :

$$E_{ab} = E_{es} + E_{cv} \qquad IV.2$$

The electrophile A here corresponds to the site of attack on the ultimate carcinogen, while the nucleophile B corresponds to the DNA base site. Here, the covalent component may be expressed as

$$E_{cv} = - FK \qquad IV.3$$

where,

$$F = \sum_{m}^{A} \sum_{n}^{B} (2C_n C_m)^2 / (E_a - E_b) \qquad IV.4$$

C_m and C_n being coefficients of atomic orbitals m and n for atoms in A and B. E_a and E_b refer to the energies of the appropriate LUMO and HOMO orbitals in A and B respectively. It may be inferred that E_{cv} would depend only on the value of the factor F, since the term K is constant for a fixed internuclear distance and depends only on this quantity through the overlap. Since the term K in the above equation is constant for a fixed internuclear distance R_{ab}, it becomes possible to make use simply of the

coefficient F to gauge the frontier orbital contribution to DNA-carcinogen reactivity.

The electrostatic term E_{es} may be expressed as

$$E_{es} = \sum_{a}^{A} \sum_{b}^{B} Q_a \, Q_b \, /DR_{ab} \qquad IV.5$$

where Q_a and Q_b are the point charges on atoms a and b summed over molecules A and B respectively, separated by a distance R_{ab} (taken constant at 3.0 A for all cases), D being the dielectric constant. At first, the vacuum phase is assumed here for simplicity in the absence of precise knowledge of D for biological media, so that D equals unity. Following the suggestion of Prof. N. Yathindra (personal communication), a D value of about 4.0 is also used as an alternative, which has been found to yield good predictions for medium range intermolecular interactions in aqueous medium.

3. *Thermodynamic Index involving adduct-formation reaction:*

Besides the above two approaches, a third, *viz.* the enthalpy of the adduct-formation reaction may also be employed in order to gauge feasibility of the reaction from the thermodynamic point of view. Unfortunately, owing to constraints in computer time here, no transition state calculations were carried out. The gas-phase enthalpy of adduct formation ΔH_{ad} is represented as in *Eqn.IV.6* below :

$$ArNH^{+} + B \text{ ------> } [ArNH\text{-}B]^{+} + \Delta H_{ad} \qquad IV.6$$

Table IV.1a: MNDO calculated FMO and ED indices for N-sites nitrenium ions*

species	Q_n	Q_{nh}	Q_{fo}	E^{**}_{lumo}
$2NA^+$	-0.033	0.102	0.139	-7.16
$4AB^+$	-0.046	0.094	0.138	-6.92
BZ^+	-0.090	0.039	0.092	-6.16
$2AF^+$	-0.063	0.073	0.114	-6.76
$2AAF^+$	-0.164	---	0.187	-6.98
MAB^+	-0.050	0.088	0.145	-6.65

Table IV.1b: MNDO calculated FMO and ED indices for C-sites (adjacent to amine group attached) of carcinogenic aromatic nitrenium ions*

species	Q_c	Q_{ch}	Q_{fo}	E^{**}_{lumo}
2NA+	0.188	0.288	0.310	-7.16
4AB+	0.153	0.273	0.286	-4.14
BZ+	0.103	0.213	0.271	-3.29
2AF+	0.153	0.272	0.241	-3.99
2AAF+	0.161	0.276	0.271	-4.19
MAB^+	0.149	0.272	0.251	-4.12

* **all indices in atomic units**

** in eV

It must be pointed out here that this chapter treats adduct formation having only the immediate product of covalent binding in mind, which is a cationic arylnitrenated DNA base. This initial product can then undergo deprotonation to yield the neutral adduct, which process is treated in Chapter Five.

IV.4 Theoretical Treatment of Product Distribution

Tables IV.Ia and IV.Ib present once again the MNDO values of the electron distribution (ED) and frontier molecular orbital (FMO) indices for the N-site and the C-site (adjacent to the imine nitrogen) respectively of various arylnitrenium ions. The ED indices are represented by the charges Q_n and Q_c, as well as by Q_{nh} and Q_{ch}, which represent the electrostatic contribution to reactivity. The FMO indices Q_{fo} and E_{lumo} represent the overlap (covalent) contribution to reactivity. A good demarcation is afforded by both FMO and ED indices for electrophilicity between these two sites. The carbon is predicted to be more reactive than the nitrogen on account of both charge as well as frontier-controlled aspects of reactivity, so that the order of electrophilic reactivity is in descending order of magnitude: C-site > N-site for all the arylnitrenium ions taken for study here.

Table IV.2: MNDO calculated FMO and ED indices, steric accessibility and molecular electrostatic potential (double helix) for different sites of DNA bases

site	Q_b^*	E_{homo}^{**}	C_x^*	SA^a	MEP^b
Oxygen sites					
O^6-G	-0.313	-11.541	0.587	2.6	-654
O^4-T	-0.330	-11.357	0.641	2.2	-612
O^2-C	-0.333	-11.626	0.508	0.0	-645
Nitrogen sites					
N^7-G	-0.146	-11.421	0.370	4.1	-683
N^2-G	-0.251	-11.822	0.622	0.0	-623
N^3-A	-0.207	-12.774	0.525	0.7	-668
N^6-A	-0.249	-11.344	0.501	0.0	-600
Carbon sites					
C^8-G	0.052	-8.853	0.450	1.0	-630
C^8-A	0.046	-8.963	0.404	0.9	-610

* indices in atomic units, ** index in eV

a in A^2 (Levery *et al* 1981)

b in kcal/mole (Pullman & Pullman 1980)

Table IV.2 gives the MNDO values for the Mulliken atomic charge Q_b, the coefficient of the appropriate HOMO C_x, the appropriate HOMO energy level E_{homo} and steric accessibility (SA), the molecular electrostatic potential minima (MEP, in double helical structure) for three oxygen sites (O^6-G, O^4-T, O^2-C), four nitrogen sites (N^7-G, N^2-G, N^6-A, N^3-A) and two carbon sites (C^8-G, C^8-A) of DNA bases. The Q_b index for the electrostatic contribution indicates a general ordering for the hard aspect of reactivity as follows: **O-sites > N-sites > C-sites.** This same ordering is not quite followed by the other indices. Perhaps a full treatment of the total overlap (covalent) contribution taking all empty orbitals in to account might change the picture here. It is thus at present hazardous to propose any ranking with respect to the soft contribution to DNA base site reactivity on the basis of the simplified indices used here for this.

It is necessary here to treat the concept of intermolecular reactivity by proposing three levels of electrophilic reactivity, and similarly for nucleophiles. These three classifications are "hard" (involving primarily electrostatic interactions, "soft" (involving primarily overlap or covalent interactions, as well as "medium" (where the electrostatic and overlap contributions are of the same order). It is here proposed that the interaction between the nitrenium ion and the DNA base site is of the **"medium-medium"**

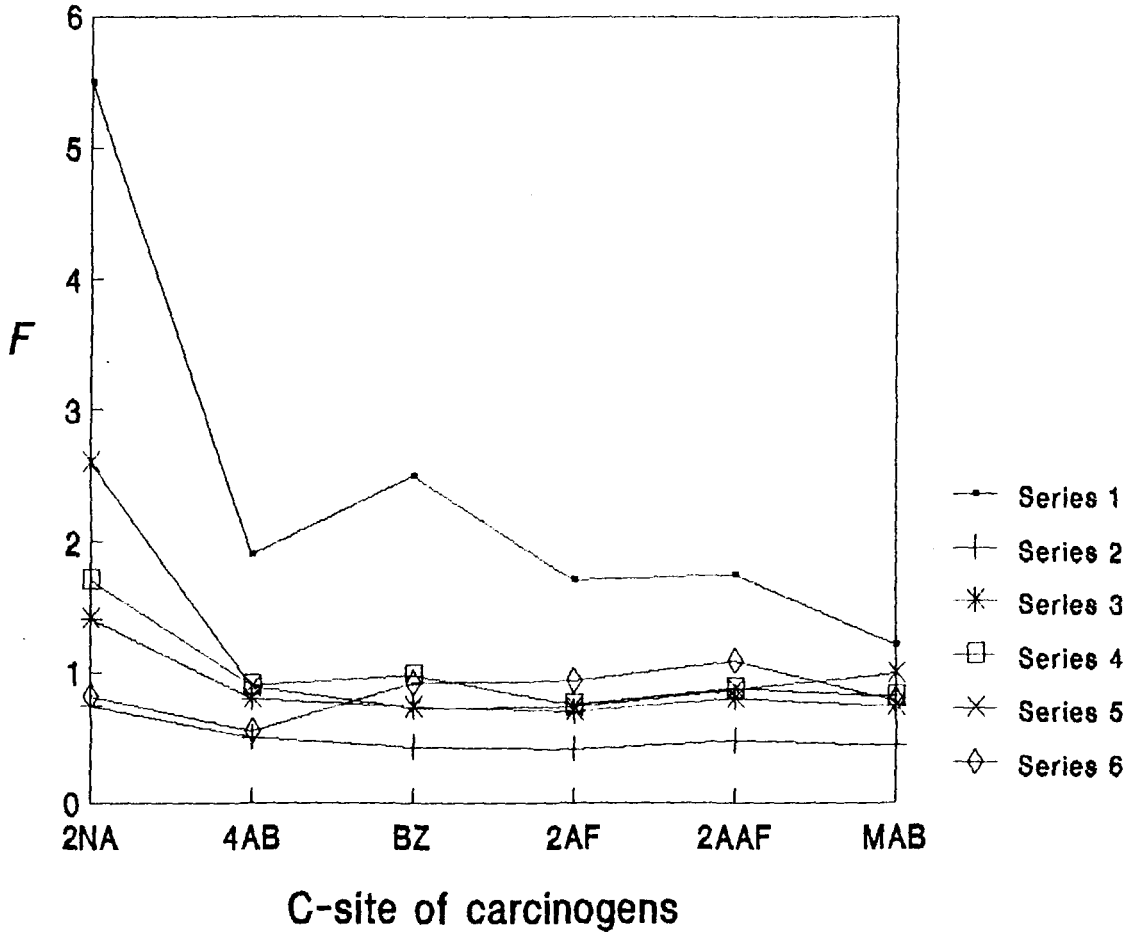


Fig.IV.3: Comparison of *F* term for C-site of carcinogens with different sites of DNA bases. series 1: N^2-G; series 2: N^7-G; series 3: N^3-A; series 4: N^6-A; series 5: C^8-G; series 6: C^8-A.

Table IV.3a: Covalent interaction (*F*) terms* for C-sites of carcinogen with different sites of DNA bases

C-sites	N^2-G	N^7-G	N^3-A	N^6-A	C^8-G	C^8-A
$2NA^+$	5.470	0.736	1.425	1.711	2.594	0.812
$4AB^+$	1.942	0.498	0.846	0.918	0.895	0.551
BZ^+	2.495	0.422	0.729	0.979	0.722	0.911
$2AF^+$	1.688	0.411	0.701	0.756	0.731	0.927
$2AAF^+$	1.746	0.473	0.805	0.874	0.860	1.082
MAB^+	1.153	0.438	0.738	0.807	0.992	0.784

Table IV.3b: Covalent interaction (*F*) terms* for N-sites of carcinogen with different sites of DNA bases

N-sites	N^2-G	N^7-G	N^3-A	N^6-A	C^8-G	C^8-A
$2NA^+$	1.065	0.399	0.613	0.768	1.538	1.171
$4AB^+$	1.001	0.385	0.595	0.717	1.326	1.012
BZ^+	0.420	0.210	0.353	0.411	0.641	0.496
$2AF^+$	0.805	0.309	0.482	0.473	1.022	0.781
$2AAF^+$	1.365	0.540	1.564	0.992	1.868	1.423
MAB^+	0.992	0.369	0.599	0.715	1.222	0.945

* all values in atomic units

kind, with both charge- and frontier-controlled aspects contributing to reactivity.

In this mode of treatment, carcinogen-DNA reactivity may be predicted by taking into account both the charge-controlled and the frontier-controlled contributions. Obviously, the charge-controlled contribution will be attractive only if the two sites in question are of opposite charge signs; repulsion is indicated if the charges on both atoms are of the same sign. Likewise, the overlap contribution will be enhanced by close proximity between the HOMO of the base and the LUMO of the cation, where a large value of the appropriate frontier atomic orbital coefficients would lead to a net lowering of energy; features like wrong orbital symmetry or a large gap between the HUMO and LUMO would tend to instability.

Table IV.3a gives the covalent interaction energy term F in atomic units (*Eq.IV.4*) for interaction of the reactive carbon site of nitrenium ions with various sites on the DNA bases. Table IV.3b presents the same for interaction of the imino nitrogen of the carcinogenic cations with various sites on DNA bases. From Table IV.3a, we see from values of the F term that the maximum frontier overlap interaction of the nitrenium carbon is invariably with the N^2-G site. This is seen to hold for all the six cases studied (Fig. IV.3). Since it is known from product structure that optimum bonding of the nitrenium carbon occurs chiefly or only

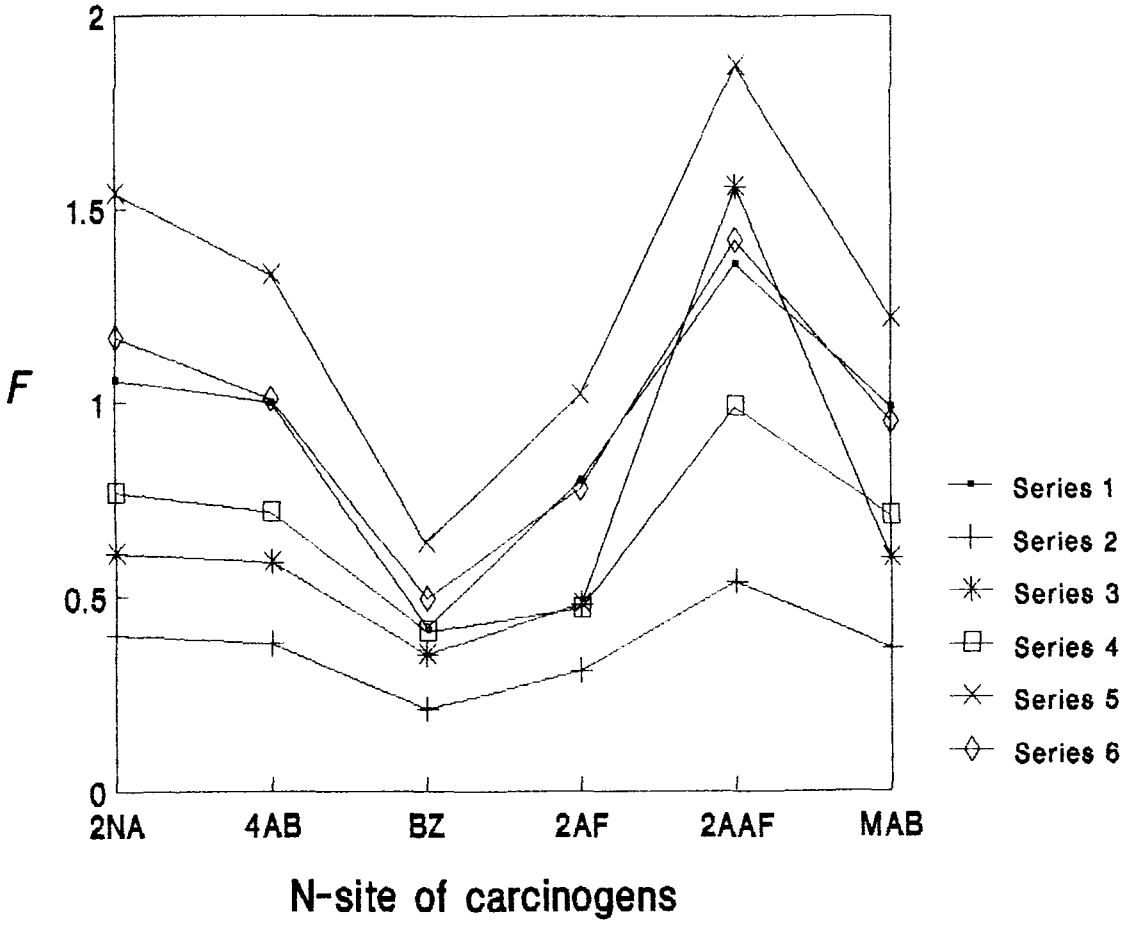


Fig.IV.4: Comparison of *F* term for N-site of carcinogens with different sites of DNA bases. series 1: N^2-G; series 2: N^7-G; series 3: N^3-A; series 4: N^6-A; series 5: C^8-G; series 6: C^8-A.

Table IV.4a: Electrostatic interaction (E_{es}) terms# for C-sites of carcinogen with different sites of DNA bases

C-sites	N^2-G	N^7-G	N^3-A	N^6-A	C^8-G	C^8-A
$2NA^+$	-7.562	-2.865	-5.680	-4.882	3.117	1.891
$4AB^+$	-7.183	-2.332	-4.582	-3.975	2.960	1.540
BZ^+	-5.584	-1.573	-3.091	-2.680	2.307	1.039
$2AF^+$	-7.154	-2.340	-4.596	-3.986	2.944	1.543
$2AAF^+$	-7.246	-2.452	-4.813	-4.174	2.988	1.616
MAB^+	-7.838	-4.559	-5.962	-4.770	2.999	1.624

Table IV.4b: Electrostatic interaction (E_{es}) terms# for N-sites of carcinogen with different sites of DNA bases

N-sites	N^2-G	N^7-G	N^3-A	N^6-A	C^8-G	C^8-A
$2NA^+$	1.045	0.607	1.192	1.034	-0.400	-0.215
$4AB^+$	1.434	0.833	1.635	1.420	-0.550	-0.297
BZ^+	2.830	1.644	3.227	2.797	-1.045	-0.582
$2AF^+$	1.989	1.156	2.269	1.967	-0.550	-0.411
$2AAF^+$	5.175	3.004	5.902	5.118	-1.984	-1.067
MAB^+	2.646	1.539	3.025	2.625	-1.012	-0.548

 # all values in kcal/mole

with the N^2-G site, this finding may be taken to indicate that the frontier-controlled aspect of reactivity contributes much here. This is seen to be particularly prominent for the case of the 2-naphthylnitrenium ion.

Likewise, from Table IV.3b, it is observed that the maximum frontier overlap interaction of the imino nitrogen of the cation is invariably with the C^8-G site, which holds true for all cases (Fig. IV.4). This fits in very well with the experimental finding that this electrophilic nitrogen binds most often to the C^8-G position during adduct formation. Here again, we can infer that the frontier-controlled aspects of chemical reactivity play a key role here.

Table IV.4a present the electrostatic interaction terms E_{es}, in kcal/mole (*Eq. IV.5*) for interactions between the electrophilic carbon of the carcinogenic cation with various DNA base sites. It is evident that while this interaction with all the nitrogen base sites is attractive, the carbon-carbon interaction between carcinogen and base sites C^8-G and C^8-A is repulsive, owing to the similarity of charge sign. This prediction is well in line with the fact that carbon-carbon bonding between carcinogen and DNA is never observed for aromatic amines. It also indicates that it is primarily the charge-controlled aspect of reactivity which precludes the formation of carbon-carbon bonds here.

Table IV.4c: Electrostatic interaction (*Ees*) terms# for C-sites of carcinogen with different sites of DNA bases (using *D* = 4.0)

C-sites	N^2-G	N^7-G	N^3-A	N^6-A	C^8-G	C^8-A
$2NA^+$	-1.89	-0.71	-1.42	-1.22	0.77	0.47
$4AB^+$	-1.79	-0.58	-1.14	-0.99	0.74	0.38
BZ^+	-1.39	-0.39	-0.77	-0.67	0.57	0.26
$2AF^+$	-1.78	-0.58	-1.15	-0.99	0.73	0.38
$2AAF^+$	-1.81	-0.61	-1.20	-1.04	0.74	0.40
MAB^+	-1.96	-1.14	-1.49	-1.19	0.75	0.41

Table IV.4b: Electrostatic interaction (*Ees*) terms# for N sites of carcinogen with different sites of DNA bases (using *D* = 4)

N-sites	N^2-G	N^7-G	N^3-A	N^6-A	C^8-G	C^8-A
$2NA^+$	0.26	0.15	0.30	0.26	-0.10	-0.05
$4AB^+$	0.36	0.21	0.41	0.35	-0.14	-0.07
BZ^+	0.71	0.41	0.80	0.70	-0.26	-0.14
$2AF^+$	0.49	0.29	0.56	0.49	-0.14	-0.09
$2AAF^+$	1.29	0.75	1.47	1.28	-0.49	-0.26
MAB^+	0.66	0.38	0.76	0.66	-0.25	-0.14

 # all values in kcal/mole

Much the same holds for the case of interaction between the imino nitrogen of the cation and the various DNA base sites, for which the E_{es} terms are presented in Table IV.4b. Here, only nitrogen-carbon interaction is found to be favourable, while the interaction between the cationic imino nitrogen and all the nitrogen base sites is repulsive. This falls well in line with the observations of experiment that adducts with nitrogen-nitrogen bonds are not found. Once again, we are led to infer that it is primarily the electrostatic component of the interaction that rules out the formation of nitrogen-nitrogen bonds during adduct formation.

The use of an assumed dielectric constant of D equal to 4.0 obviously does not change the picture (Table IV.4c and Table IV.4d). The value of D = 80 cannot be used here, since this value is for bulk water, while the much smaller value of 4.0 or so points to the existence of bare space in between individual water molecules, which is significant only for microscopic level calculations.

Table IV.5 compares the enthalpies of adduct formation given by ΔH_{ad} (*Eq. IV.6*), with values for the C^8-G and N^2-G cationic adducts. Examination of the data reveals that the thermodynamic stability of the cationic adduct with respect to the DNA base site is invariably C^8-G > N^2-G regardless of the aromatic amine. As has been discussed, this is just what is found to be the case

Table IV.5: Calculated Enthalpiies[#] (ΔH_{ad}) for C^8-G and N^2-G Cationic Adduct Formation

C^8-G	ΔH_{ad}	N^2-G	ΔH_{ad}
$2NA^+$	-31.8	$2NA^+$	2.2
$4AB^+$	-29.2	$4AB^+$	10.8
BZ^+	-22.5	BZ^+	11.8
$2AF^+$	-22.1	$2AF^+$	14.4
$2AAF^+$	-31.7	$2AAF^+$	-4.3
MAB^+	-28.6	MAB^+	5.5

all values in kcal/mole

experimentally in most cases. This theoretical indicator thus points towards establishment of the order obtained by experiment.

IV.5 Conclusions

All these theoretical indicators point towards the specificity of bonding between the N-site of the nitrenium ion and the C^8-G site of guanine, as well as the specificity of bonding between the reactive C-site of the carcinogenic cation and the N^2-G site of guanine, thus furnishing good rationalisations well in line with known experimental findings.

Reactive sites on the ultimate carcinogen included the nitrenium N-site as well as the carbon just proximate to the C-N bond. It was found that while nitrogen-nitrogen or carbon-carbon affinity were not the best favoured, carbon-nitrogen and nitrogen-carbon affinity was appreciably high, for which the electrophile-nucleophile interaction is of the "**medium-medium**" type, with both charge- as well as frontier-controlled aspects operating.

Lastly, the calculated enthalpies of formation of the cationic adducts also lead to the inference that the N^2-G adduct formation would be favoured over C^8-G adduct formation, as experiment has found to be generally true.

Chapter V

CHAPTER V

STRUCTURAL CHANGES IN DNA MODIFIED BY AROMATIC AMINES

V.1 DNA Alterations Induced by Carcinogens

Carcinogens are known to alter natural DNA in a variety of ways. The most obvious alteration is, of course, the addition of the xenobiotic carcinogen moiety to the native DNA. This binding may be covalent or non-covalent. Evidence exists for the non-covalent binding of carcinogens to DNA *via* weak, loosely-bonded interactions (*eg.* van der Waals forces), as could well be the case for certain inorganic carcinogens like asbestos. By far, however, the vast majority of chemical carcinogens are believed to operate through strong covalent binding to DNA (specifically the DNA base residues) as has been dealt with in the last chapter for aromatic amines. Besides this primary chemical modification, other concomitant or resultant alterations include the following :

(1) Proton abstraction from carcinogen-DNA adducts : The immediate product of ultimate carcinogen attack on DNA bases in most cases possesses a net positive charge owing to the cationic nature of the electrophilic attacking moiety. Loss of a proton furnishes a neutral deprotonated modified base residue. Such changes have been studied extensively for alkylated DNA bases and nucleosides, where in some cases the deprotonation reaction is intimately connected to the manifestation of mutagenic and even carcinogenic responses. For aromatic amines, the DNA adducts have been characterised in numerous cases, as summarised by Garner *et al* (1984), where the chemical structures at biological pH have been shown as deprotonated and neutral. In these cases, the proton may be lost from the DNA base residue or even from the carcinogen moiety itself.

(2) Other protic changes : Tautomerism in carcinogen-DNA adducts provides an alteration where a proton is transferred from one bound site to another. This could occur spontaneously within the newly formed cationic adduct itself, or occur in the neutral adduct too. Tautomerism in normal DNA has been invoked as a likely mechanism for spontaneous mutagenesis, and this concept is explored here for carcinogen-DNA adducts as well.

(3) Loss of the modified base : Observations have been noted where the carcinogen-DNA adduct undergoes loss of the modified

base moiety through depurination or depyrimidination (the former being most commonly observed). Certain alkylated DNA nucleosides are prone to spontaneous depurination, creating an abasic site which is a non-informational lesion. Abasic sites are potential inducers of non-specific base substitutional mutations. Their likely role for point mutagenesis and oncogene activation in aromatic amine carcinogenesis is discussed in Chapter Six.

(4) DNA conformational changes : In the case of bulky carcinogens, their binding to DNA would be expected to exert appreciable steric distortions, resulting in accomodations of various kinds within the double helix. These include appreciable changes in nucleic acid conformation, *viz.* alteration in conformation at the glycosyl bond, rotations of the backbone residues, and possible changes in sugar puckering. The covalent attachment of bulky carcinogens such as 2-acetylaminofluorene (AAF), 2-naphthylamine and others do present steric problems, and depending on the sites of the substitutions, may be associated with major distortions in the native conformation of nucleic acids (Grunberger & Weinstein 1978).

These changes are best illustrated on drawings of base-displacement minimum energy conformation of dCpdG-AAF and in a computer-generated stereoscopic display of a double-stranded DNA fragment (Hingerty & Broyde 1982).

Experimental evidence of the base displacement model has been obtained from proton magnetic resonance and CD spectra of modified oligonucleotides (Santella *et al* 1980; Sage & Leng 1980). The technique of electric dichroism was then used to determine the orientation of the covalently bound fluorene ring to the long axis of the DNA. The results clearly indicated that in the case of DNA-AAF the fluorene ring lies almost perpendicular to the helix axis, the angle being about 80^{o} (Hingerty & Broyde 1982). Since there are differences in the steric aspects connected with modification of the C^{8} and N^{2} positions of G, differences also exist between the conformational distortions in the DNA helix associated with these two types of adducts. It appears from experiment that in contrast to the C^{8} adduct, substitution of AAF on the N^{2} position of guanine does not produce a major change in conformation of the DNA helix. Although the precise conformation of the helix at the latter sites has not been determined, model-building studies indicate that the N^{2}-position is in contrast to the C^{8} position of guanine, readily susceptible to chemical modifications, and the fluorene residue could simply occupy the minor groove of the DNA helix. Thus, the base displacement model may apply only to to the C^{8}-G adduct and not to the N^{2}-G adduct of AAF (Singer & Grunberger 1983).

V.2 Proton Lability and Acidity in C^8-G and N^2-G Adducts

With evidence for the possible carcinogenic role of protic changes in carcinogen-DNA adducts as established for alkylating agents and N-nitroso compunds (Duncan 1989; Venkateswarlu 1995), the question arises as to what likely role protic changes might have for carcinogenesis by polycyclic aromatic amines. The protic change involving proton abstraction is discussed in this section, while the protic change involving tautomerism is discussed in Sec. V.3 below.

V.2.1 Proton abstraction from carcinogen-DNA adducts

One possible protic change that can occur in a carcinogen-DNA adduct is that of abstraction or loss of a proton from the cationic adduct. Such a change has been invoked (Duncan & Davies 1989; Lyngdoh 1992) to furnish a molecular basis for the generation of point mutations by alkylating agents. This survey was carried out in order to see if such a promutagenic mechanism might be operational for arylnitrenated DNA base adducts too. The survey encompassed a number of potentially labile or acidic protons in the cationic adducts, with a view to rate them in order of their relative proton labilities and proton acidities. Here, the concept of proton lability was distinguished from that of

proton acidity, the former being a kinetic definition and the latter a thermodynamic one. The twelve different cationic adducts between guanine and the arylnitrenium moiety which were considered in the earlier chapters were included here too.

V.2.2 Theoretical indices for proton lability and acidity

The MNDO SCF-MO optimised geometries of the carcinogen-DNA adducts were used to derive the following indices for proton lability and for proton acidity for various protons present :

1. Proton lability indices : These were deduced from the wave-function for the cationic adducts, and refer primarily to the reaction rates involved in abstraction of the various protons. In consideration of the large negative values for enthalpies of deprotonation (with hydrated hydroxide anion as candidate deprotonator) as calculated for a few representative cases (see Sec. V.2.4 below), the Hammond postulate was invoked to propose a closeness in energy and structure between the reactants and the transition state. This led to use of the reactant cationic adduct as a plausible furnisher of information regarding reactivity of the various protons concerned (instead of the transition state, which was computationally not feasible to calculate here). The proton lability indices used here thus derive from quantities

connected with electrophilic character of the protons, and include the following :-

(a) the Mulliken charge Q_h on the relevant protons

(b) the bond strength between the concerned proton and the atom to which it is attached, given by the Wiberg index W_{xh}

(c) the frontier MO interaction term T_{fo} for covalent affinity between the candidate deprotonator and the proton of interest, as given by *Eq. V.1* below :

$$T_{fo} = C_s^2/(E_1 - E_w) \qquad V.1$$

where C_s and E_1 are the hydrogen *1s* atomic orbital coefficient and energy level of the LUMO involving the hydrogen, while E_w is the energy of the HOMO of the water molecule.

(d) the frontier electron density Q_{fo} on the hydrogen atom, given by the square of the 1s AO coefficient in the lowest empty MO significantly involving the proton concerned

2. Proton acidity indices : These were represented by the enthalpy change for the following equilibria of *Eqns. V.2* and *V.3(a-c)* below, where the former ($\triangle H_p$) is the negative of the gas-phase proton affinity, and the latter the enthalpy of deprotonation using a water molecule ($\triangle H_w$), the hydrated hydroxide anion $[OH(H_2O)_3]^-$ ($\triangle H_{aq}$), and the hydroxide anion OH^- ($\triangle H_{oh}$), as deprotonators respectively.

Table V.1a: Indices for N^1 Proton lability for C^8-G Adducts*

species	Q_h	ω_{xh}	Q_{fo}	T_{fo}
2NA	0.233	0.898	0.199	0.390
4AB	0.234	0.897	0.193	0.360
BZ	0.236	0.896	0.146	0.272
2AF	0.236	0.896	0.162	0.302
2AAF	0.234	0.897	0.218	0.399
MAB	0.235	0.897	0.193	0.369

Table V.1b: Indices for N^2 Proton lability for C^8-G Adducts*

species	Q_h	ω_{xh}	Q_{fo}	T_{fo}
2NA	0.233	0.907	0.141	0.337
4AB	0.233	0.907	0.160	0.383
BZ	0.232	0.908	0.151	0.360
2AF	0.234	0.907	0.165	0.392
2AAF	0.232	0.907	0.099	0.251
MAB	0.234	0.907	0.149	0.357

Table V.1c: Indices for N^2 Proton lability for C^8-G Adducts*

species	Q_h	ω_{xh}	Q_{fo}	T_{fo}
2NA	0.254	0.899	0.103	0.254
4AB	0.253	0.900	0.104	0.249
BZ	0.253	0.900	0.099	0.237
2AF	0.254	0.899	0.106	0.251
2AAF	0.254	0.899	0.081	0.194
MAB	0.254	0.899	0.101	0.242

* All values in atomic unit

Table V.1d: Indices for N^9 Proton lability for C^8-G Adducts*

species	Q_h	w_{xh}	Q_{fo}	T_{fo}
2NA	0.264	0.881	0.167	0.277
4AB	0.255	0.885	0.141	0.237
BZ	0.267	0.880	0.196	0.330
2AF	0.257	0.885	0.152	0.256
2AAF	0.265	0.880	0.139	0.254
MAB	0.254	0.885	0.153	0.256

Table V.1e: Indices for C^8 Proton lability for C^8-G Adducts*

species	Q_h	w_{xh}	Q_{fo}	T_{fo}
2NA	0.105	0.922	0.264	0.530
4AB	0.109	0.924	0.374	0.754
BZ	0.111	0.921	0.300	0.604
2AF	0.099	0.917	0.203	0.410
2AAF	0.119	0.917	0.361	0.715
MAB	0.118	0.923	0.222	0.445

Table V.1f: Indices for N_{amine} Proton lability for C^8-G Adducts*

species	Q_h	w_{xh}	Q_{fo}	T_{fo}
2NA	0.177	0.927	0.175	0.281
4AB	0.174	0.931	0.182	0.297
BZ	0.157	0.939	0.255	0.415
2AF	0.145	0.944	0.216	0.355
2AAF	-	-	-	-
MAB	0.175	0.924	0.156	0.251

* All values in atomic unit

$$[ArNHBH]^+ \text{------>} [ArNHB] + H^+ + \Delta H_p \qquad V.2$$

$$[ArNHBH]^+ + H_2O \text{------>} [ArNHB] + H_3O^+ + \Delta H_w \qquad V.3a$$

$$[ArNHBH]^+ + [OH(H_2O)_3]^- \text{-----> } [ArNHB] + 4H_2O + \Delta H_{aq} \qquad V.3b$$

$$[ArNHBH]^+ + HO^- \text{------>} [ArNHB] + H_2O + \Delta H_{oh} \qquad V.3c$$

Here, BH refers to the base with the proton concerned, while $ArNH^+$ refers to the arylnitrenium ion moiety, so that $[ArNHBH]^+$ and [ArNHB] represent the cationic and deprotonated forms of the carcinogen-guanine adducts.

V.2.3 Proton labilities compared

Tables V.1(a-f) and Tables V.2(a-f) present the MNDO values of the frontier orbital and electron distribution indices for electrophilic reactivity (kinetic lability) of various protons (the N^1, N^2, N^9, C^8 protons of guanine and the amino proton of the arylnitrenium moiety) as calculated for the six C^8-G and the six N^2-G cationic adducts respectively, each denoted by the symbol for the parent amine. Both the 2-amino protons of the C^8-G adducts were studied (Tables V.1b and V.1c), and likewise for the N^2-G adducts (Tables V.2b and V.2c).

It is evident that the charge-controlled aspects of proton reactivity (as given by the electron distribution indices Q_h and W_{xh}) do not lead to the same inferences and orderings as given by

Table V.2a: Indices for N^1 Proton lability for N^2-G Adducts*

species	Q_h	ω_{xh}	Q_{fo}	T_{fo}
2NA	0.215	0.902	0.294	0.507
4AB	0.210	0.904	0.274	0.484
BZ	0.220	0.901	0.268	0.461
2AF	0.209	0.905	0.281	0.484
2AAF	0.217	0.902	0.283	0.484
MAB	0.216	0.902	0.278	0.484

Table V.2b: Indices for N^2 Proton lability for N^2-G Adducts*

species	Q_h	ω_{xh}	Q_{fo}	T_{fo}
2NA	0.206	0.928	0.169	0.385
4AB	0.200	0.926	0.212	0.484
BZ	0.208	0.927	0.132	0.311
2AF	0.198	0.928	0.115	0.254
2AAF	0.221	0.917	0.130	0.286
MAB	0.221	0.920	0.147	0.323

Table V.2c: Indices for N^2 Proton lability for N^2-G Adducts*

species	Q_h	ω_{xh}	Q_{fo}	T_{fo}
2NA	0.221	0.922	0.205	0.461
4AB	0.229	0.920	0.196	0.459
BZ	0.221	0.921	0.183	0.403
2AF	0.227	0.919	0.118	0.277
2AAF	0.227	0.920	0.110	0.247
MAB	0.223	0.923	0.216	0.484

* All values in atomic unit

Table V.2d: Indices for N^9 Proton lability for N^2-G Adducts*

species	Q_h	w_{xh}	Q_{fo}	T_{fo}
2NA	0.246	0.890	0.150	0.334
4AB	0.246	0.890	0.146	0.330
BZ	0.244	0.891	0.171	0.374
2AF	0.246	0.890	0.156	0.346
2AAF	0.245	0.891	0.103	0.226
MAB	0.245	0.891	0.062	0.122

Table V.2e: Indices for C^8 Proton lability for N^2-G Adducts*

species	Q_h	w_{xh}	Q_{fo}	T_{fo}
2NA	0.162	0.927	0.218	0.392
4AB	0.162	0.927	0.221	0.408
BZ	0.160	0.928	0.257	0.466
2AF	0.161	0.927	0.201	0.369
2AAF	0.160	0.927	0.154	0.281
MAB	0.160	0.928	0.156	0.286

Table V.2f: Indices for C_{amine} Proton lability for N^2-G Adducts*

species	Q_h	w_{xh}	Q_{fo}	T_{fo}
2NA	0.084	0.957	0.127	0.277
4AB	0.074	0.955	0.179	0.380
BZ	0.099	0.947	0.185	0.360
2AF	0.079	0.956	0.192	0.399
2AAF	0.085	0.951	0.168	0.339
MAB	0.082	0.947	0.096	0.198

* All values in atomic unit

the frontier-controlled indices (Q_{fo} and T_{fo}). This may be taken to mean that the ratings for the hard contribution to proton electrophilicity do not correspond to the ratings provided by the soft contribution.

For all the C^8-G adducts, the "hard" indices Q_h and W_{xh} predict that the N^9 proton possesses the maximum hard contribution to proton lability. The "soft" indices Q_{fo} and T_{fo}, on the other hand, predict that it is the C^8 proton of these adducts that would possess the maximum soft contribution towards proton lability and electrophilicity. These inferences hold for each of the six cases examined. Since the hard and soft effects seem to operate in opposite directions, as it were, it is difficult at this level of calculation to furnish predictions as to which proton would be the most labile kinetically. This is because the relative weightages of the hard and soft contributions towards net reactivity is not directly obtainable from these calculations.

Elementary mechanistic and structural considerations, however, suggest that it is the C^8 proton, and not the N^9 proton, that would depart most readily in the case of the adducts modified at the C^8-G position. If this indeed be the case, it then follows that the "soft" contribution to proton lability is what decides the outcome here for the C^8-G adducts, not the "hard" aspect.

Note that the N^1 proton, whose abstraction is of crucial significance for mutagenesis and carcinogenesis by alkylating agents, is not here predicted to be the most labile either by "hard" or "soft" considerations. This is also seen to hold for the two N^2 protons. This thereby casts doubt (at least from the kinetic point of view) upon the role of Watson-Crick proton abstraction (whether N^1 or N^2) for mutagenesis or carcinogenesis by polycyclic aromatic amines, at least so far as the C^8-G adducts are concerned.

For the adducts modified at the N^2-G site, the "hard" indices predict that the N^9- proton would have the maximum hard contribution towards electrophilicity. The "soft" indices predict that the N^1- proton would possess the maximum soft contribution towards proton reactivity. Here again, all the six cases covered yield the same inferences. Since the weightages of the hard and soft contributions to net proton lability are not known, it is not possible to predict which proton would in fact have the best combination of both aspects.

Here too, elementary considerations prove helpful by proposing that, in fact, one of the N^2- protons would be the most ready to be abstracted. It may be seen from the values of the indices that both these protons are characterised by possessing an intermediary status among the various protons with regard to both "hard" and

"soft" contributions. If it be indeed true (as is most likely so) that the N^2- protons are kinetically the most labile, we can infer that this is so because they possess the best combinations of both "hard" and "soft" aspects to reactivity. This implies that both aspects play significant roles for deciding the issue here, unlike in the case of the C^8-G adducts, (where proton lability appeared to be influenced primarily by the soft aspect).

The nature of the atom to which the labile proton is bonded is of relevance here. For the C^8-G adducts, assuming highest lability for the C^8 proton, the soft aspect of reactivity plays the primarily role owing to the small electronegativity difference between carbon and hydrogen. For the N^2-G adducts, assuming highest lability for the N^2- protons, we can infer that the hard aspect of reactivity begins to play a role here owing to the increased electronegativity difference in this case (between nitrogen and hydrogen). These observations may be extrapolated to a hypothetical similar system having the labile proton bonded to oxygen, in which case the hard aspect would be expected to play a dominant role in determining proton lability owing to the even higher electronegativity difference here (in this case between oxygen and hydrogen).

The above predictions and findings unfortunately do not have immediate relevance for current experimental results, where data

has yet to be established for relative proton labilities in these systems. Under conditions of appropriate *pH* (corresponding to the *pKa* value of the system), proton labilities could be compared through deuterium exchange (the kinetic isotope effect). Till then, these calculations remain purely predictive in value, but have the advantage of being backed by chemical intuition. Whether lability orderings compare well with acidity orderings remains to be seen, as is discussed in the next sub-section.

V.2.4 Proton acidities compared

This comparison of proton acidities in the cationic carcinogen-DNA adducts is undertaken with a view to understanding the protic acid-base equilibrium involved in the thermodynamically stable carcinogen-DNA adduct species. The chief inquiry here pertains to which proton is abstracted upon attainment of equilibrium. The protons of interest here are :

(a) the C^8- proton for the C^8-G adducts owing to the maximum lability associated with them (as just seen earlier)

(b) the N^2- protons for the N^2-G adducts for the same reason

(c) the N^1- proton for both C^8-G adducts and N^2-G adducts owing to the prime role that abstraction of this proton plays for other classes of carcinogen-DNA adducts (*eg.* alkylated DNA bases).

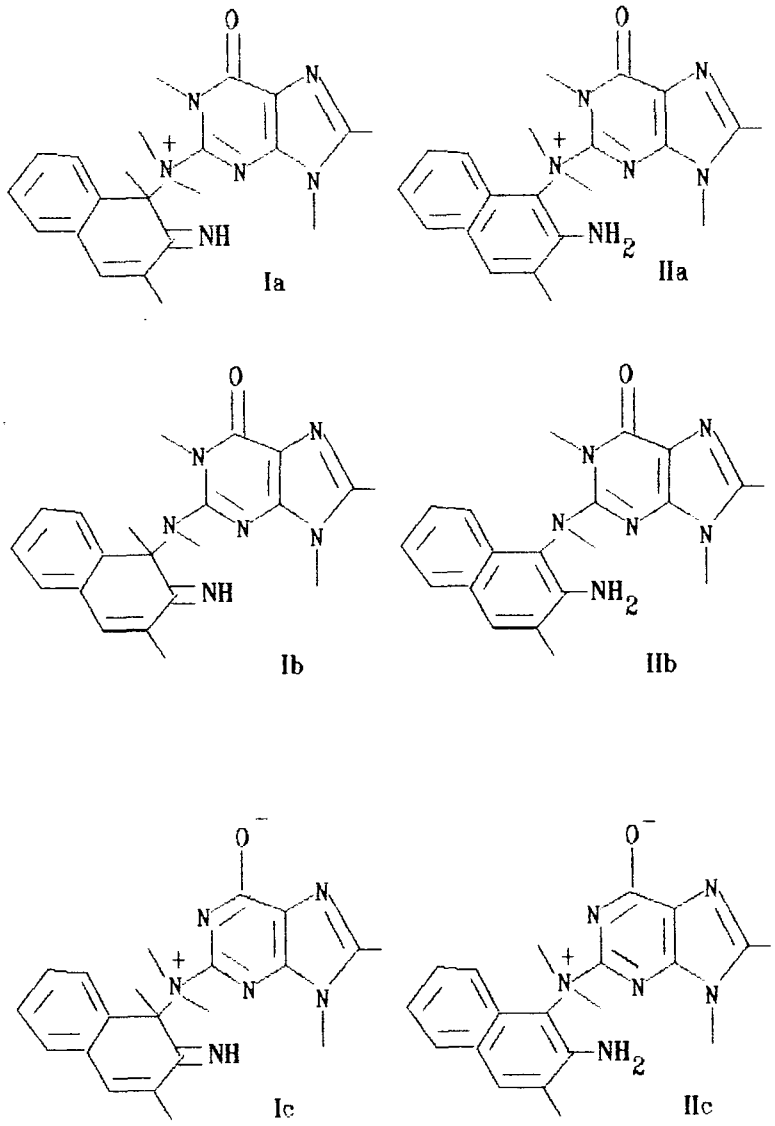


Fig.V.1: Different tautomers of N^2-G cationic and Neutral adducts with 2NA. I (a,b,c): imine forms; II (a,b,c): amine forms.

Apart from concluding which proton is the most acidic, this study would also conclude whether the proton with maximum acidity and that with maximum lability are identical or not.

For the C^8-G cationic adducts, only one tautomer is proposed. For the cationic N^2-G adducts, however, two tautomers are here advanced as possible, so that calculation of proton acidity would have to reckon with both. These are the form (I) with the arylnitrenium moiety existing in its imino form (Ia in Fig, V.1), and the form (II) with this present in amino form (IIa in Fig. V.1). It is to be noted that the N^2-G cationic adduct in its imine form (I) is the immediate product of arylnitrenation at the N^2-G site through the reactive C-atom of the carcinogen moiety.

Now, the neutral C^8-G adducts may be N^1-deprotonated or C^8-deprotonated, as suggested above. Likewise, the neutral N^2-G adducts may be N^1-deprotonated or N^2-deprotonated. However, for both the N^1-deprotonated and the N^2-deprotonated N^2-G adducts (neutral), two tautomeric possibilities exist, where the tautomeric transition involves transfer of a proton from the binding C-atom of the arylnitrenium moiety to the imino group of the same. Thus, the N^2-deprotonated species may exist as two neutral tautomers (Ib and IIb in Fig. V.1), while the N^1-deprotonated adduct has the tautomers Ic and IIc of Fig. V.1. The (I) and (II) forms of the neutral adducts correspond to the forms (I) and (II) of the cationic adducts.

Table V.3: Enthalpies of different deprotonation reactions for C^8-G adducts*

	C^8-proton			N^1-proton		
	ΔH_p	ΔH_w	ΔH_{aq}	ΔH_p	ΔH_w	ΔH_{aq}
2NA	209.9	37.9	-187.1	223.2	51.1	-173.9
MAB	195.7	23.6	-201.3	221.7	49.6	-188.1

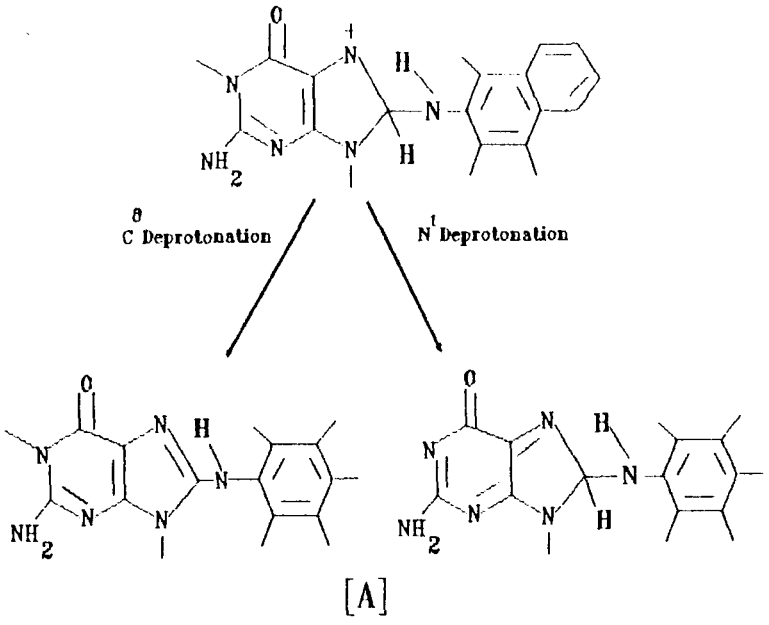
Table V.4: C^8-deprotonation enthalpies for C^8-G cationic adducts*

species	ΔH_p	ΔH_w	ΔH_{aq}	ΔH_{oh}
2NA	209.9	37.9	-187.1	-212.4
4AB	204.5	32.4	-192.5	-217.8
BZ	197.7	25.6	-199.4	-224.7
2AF	205.7	33.6	-191.4	-216.6
2AAF	209.0	36.9	-188.1	-213.8
MAB	195.7	23.6	-201.3	-226.6

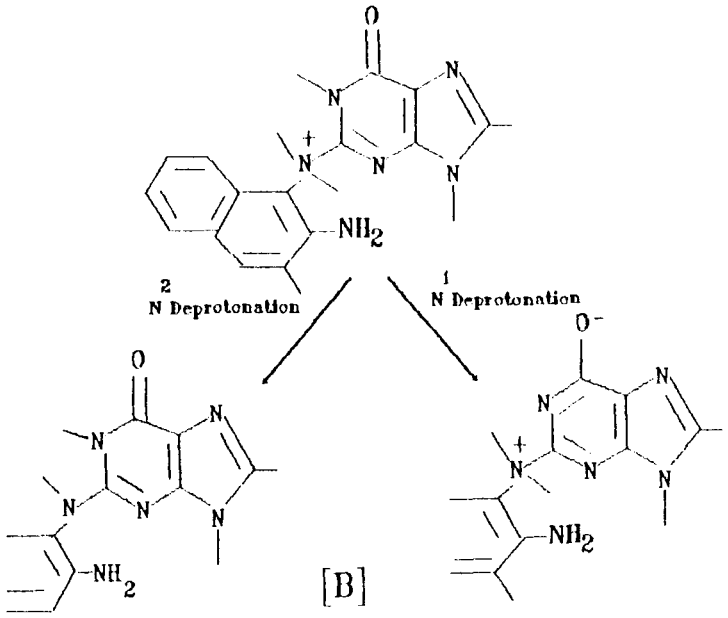
Table V.5: Enthalpiies (ΔH_{ta}) for tautomeric changes from (I) to (II) form for cationic, N^1-deprotonated and N^2-deprotonated species of N^2-G adducts*

species	cationic ΔH^+_{ta}	N^1-deprot. ΔH^o_{ta}	N^2-deprot. ΔH^o_{ta}
2NA	-18.4	-14.9	-06.0
MAB	-24.2	-23.4	-25.4

* All values in kcal/mole



[A]



[B]

Fig.V.2: Deprotonation reactions for 2NA cationic adducts.
[A]: C^{8}-G adduct; [B]: N^{2}-G (II) adduct.

The enthalpy index ΔH_{ta} (from the MNDO SCF MO method) is a measure of thermodynamic facility for this rearrangement of the cationic and neutral N^2-G adducts (which hold for N^1-deprotonation as well as N^2-deprotonation) :

N^2-G Cationic (I) ----> N^2-G Cationic (II) + ΔH^+_{ta} *V.4*

N^2-G Neutral (I) ----> N^2-G Neutral (II) + ΔH^o_{ta} *V.5*

(1) Deprotonation of C^8-G adducts :

Table V.3 presents the MNDO calculated enthalpy indices concerning C^8- and N^1-proton acidities for the cationic C^8-G adducts formed by 2-napthylamine (2NA) and methylaminoazobenzene (MAB). The deprotonation reactions are presented in Fig. V.2 (for the 2NA adducts). As discussed earlier, proton acidity here is represented by the negative of the proton affinity, as well as by the enthalpies of deprotonation with water, hydrated hydroxide anion and simple hydroxide anion as the proton acceptors. These calculations then lead to choice of the more acidic proton in each case (C^8- or N^1-proton).

The results of Table V.3 lead to the prediction that the C^8-proton would be by far the more acidic one over the N^1-proton. The difference in acidity between the C^8-proton and the N^1-proton is about 13.2 Kcal/mole for the 2NA adduct, and about 26.0

kcal/mole for the MAB adduct. Thus, the C^8-G adducts for 2NA and MAB are predicted to undergo loss of the C^8-proton rather than the N^1-proton. Upon comparing these results with those concerning proton lability of the C^8-G adducts, it is thus predicted that the C^8-proton would be simultaneously the most labile as well as the most acidic for the products of guanine C^8-arylnitrenation.

This inference that the C^8-proton is the more acidic is then extended to include all six adducts, for which the C^8-deprotonation enthalpies are given in Table V.4. The range of values for the enthalpy indices are about 196 to 210 kcal/mole for the bare deprotonation, about 24 to 38 kcal/mole for the ΔH_w index, about -187 to -201 kcal/mole for the ΔH_{aq} index, and about -212 to -226 kcal/mole for the ΔH_{oh} index. The trend of values is thus seen to range through from large positive values (for the ΔH_p index) to large negative values (for the ΔH_{oh} index). Since deprotonation at biological *p*H is assumed facile, the actual value of the free energy of deprotonation would have to be negative, *viz.* somewhere between the value ranges for the ΔH_w and ΔH_{oh} indices. The use of solvent phase simulation could lead to a better approach to the actual energetics involved here.

(2) Deprotonation of the N^2-G adducts :

Deprotonation here may involve the N^1-proton or the N^2-proton, (Fig. V.2) as described above. The cationic adduct for both is

Table V.6: Enthalpies of different deprotonation reaction for N^2-G (II) adducts*

	N^2-proton			N^1-proton		
species	ΔH_p	ΔH_w	ΔH_{aq}	ΔH_p	ΔH_w	ΔH_{aq}
2NA	198.3	26.2	-198.8	231.9	59.8	-165.1
MAB	189.5	17.4	-207.7	233.5	61.4	-174.0

Table V.7: N^2-deprotonation enthalpies for N^2-G (II) adducts*

species	ΔH_p	ΔH_w	ΔH_{aq}	ΔH_{oh}
2NA	198.3	26.2	-198.8	-224.1
4AB	197.7	25.6	-199.4	-224.6
BZ	189.9	17.9	-207.2	-232.4
2AF	189.1	17.0	-208.0	-233.2
2AAF	185.5	13.5	-211.6	-236.8
MAB	189.5	17.4	-207.7	-232.9

Table V.8: Enthalpies of keto-enol tautomerism for C^8-G, N^2-G (II) neutral adducts*

C8-G adducts	ΔH_{ke}	N2-G adducts	ΔH_{ke}
2NA	-5.62	2NA	-4.73
4AB	-5.50	4AB	-4.82
BZ	-5.32	BZ	-6.09
2AF	-3.50	AF2	-4.66
2AAF	-6.07	AAF2	-5.04
MAB	-5.44	MAB	-6.27

* All values in kcal/mole

the same, although having the two tautomeric possibilities Ia and IIa (Fig. V.1). The neutral N^2-deprotonated adduct may be Ib or IIb, while the N^1-deprotonated adduct may be form Ic or IIc (Fig. V.1).

In order to establish which species are actually involved in N^2-deprotonation here, the stabler among the tautomeric forms (I) and (II) for both cationic and neutral species have to be ascertained in each case. Table V.5 presents the values of the tautomeric facility index ΔH_{ta} for the N^2-G adducts of 2NA and MAB, with values given for the cationic case, the N^1-deprotonated case, and the N^2-deprotonated case.

It is immediately apparent upon inspection that the stabler tautomer for both cationic and neutral adducts is represented by the form (II) in each case. Thus, a more genuine approach to determining proton acidities would have to consider only the (II) forms of both cationic and neutral N^2-G adducts.

Table V.6 now presents the N^2- and N^1-deprotonation enthalpies for the N^2-G adducts of 2NA and MAB, all considered in terms of only the stabler (II) forms. The enthalpy indices correspond to bare deprotonation (ΔH_p), and water and hydrated hydroxide anion as deprotonators (ΔH_w and ΔH_{aq}). It is obvious that N^2-deprotonation is favoured for both cases over N^1-deprotonation, where the difference in deprotonation enthalpy is 33.6 kcal/mole

for the 2NA case, and 41 kcal/mole for the MAB case. The choice for the more acidic proton (the N^2-proton) is even more clear here for the N^2-G adducts than for the C^8-G adducts (where the deprotonation enthalpy differences were just 13.2 and 26.0 kcal/mole for 2NA and for MAB). The corollary conclusion here is that the most acidic proton and the most labile proton for the N^2-G adducts would be the same, *viz.* the N^2-proton.

Table V.7 then extends this finding to all the six cases covered and presents the four deprotonation enthalpies ΔH_p, ΔH_w, ΔH_{aq} and ΔH_{oh} for each case. The gamut of ranges of values for each index follows trends similar to those given by the C^8-G adducts.

(3) Conclusions :

From these calculations, it may be inferred or predicted that the C^8-proton of the C^8-G adducts would be the most labile and acidic, while it is the N^2-proton of the N^2-G adducts that would be most labile and acidic. In other words, for both cases, it is the proton present right upon the binding site between DNA and carcinogen that emerges as the most acidic and labile. This is due to the formal positive charge upon the binding site in each case.

V.3 Keto-Enol Tautomerism in Carcinogen-DNA Adducts

V.3.1 Background

In the Watson-Crick model of DNA, the bases in deoxyguanosine and deoxycytidine must adopt the keto-amino tautomer in order to form the proper complementary pair. In principle, this is not the only form possible (Szczepaniak & Szczesniak 1987). There exists a finite possibility under natural conditions of temperature and pressure for existence of minor tautomeric forms. These tautomers have been suggested to have transient mutagenic properties (for inducing base substitutions), and believed to play a role for spontaneous mutagenesis, carcinogenesis and aging.

Ab initio quantum mechanical calculation (Latazka *et al* 1986) have predicted that for the guanine monomer, the keto-amino and enol-amino tautomers have small-order energies of interconversion. For the case of DNA bases alkylated at the O^6-G and O^4-T sites, the molecules exist at neutral or biological pH in a "frozen" tautomeric form which possesses mutagenic potential, because of the absence of the N^1-G or N^3-T protons (Duncan & Davies 1989; Lyngdoh 1993).

Spectroscopic studies suggest that monomeric molecules in inert environment exist in both keto-amino and enol-amino tautomeric forms (Sheina *et al* 1985; Szczesniak *et al* 1984; Nowak *et al* 1980). In contrast to the lack of direct evidence for tautomers of

common bases, at the polymer level the rare tautomers can be detected using UV spectroscopy (Fresco *et al* 1980).

Upon conversion of the normal keto form of guanine to its enol form, the Watson-Crick (N^1-G) proton shifts to the O^6-G site,which drastically hampers the normal Watson-Crick hydrogen bonding scheme, besides facilitiating aberrant base-pairing. This process would lead to a point mutation eventually because of mismatching of the bases, where thymine substitutes for cytosine in the normal GC pair.

The possibility is advanced here that structural features of the carcinogen-DNA adducts of aromatic amines might lead to the facile adoption of the keto tautomer of guanine, where the N^1-G proton shifts to the O^6-G site, thereby disrupting normal Watson-Crick base-pairing. The C^8-G and N^2-G adducts in their neutral form are both considered here in the context of this possibility.

V.3.2 Methodology

The MNDO SCF-MO method with full geometry optimisation was used to generate the equilibrium structures and wave-functions for the keto and the enol forms of the C^8- and N^2-G adducts. Note that in this context, only the neutral (deprotonated) forms of the adducts are considered. The enthalpies ΔH_{ke} of the formal reaction process shown in *Eq. V.6* below relate to the energies of

tautomeric shift of the keto to the enol form {where, ΔH_{ke} = {ΔH_f(enol) - ΔH_f(keto)}.

Keto form ----------> Enol form + ΔH_{ke} V.6

V.3.3 Results and discussion

Table V.8 gives the enthalpy index ΔH_{ke}, the criterion that determines the facility of tautomeric shift of the N^1-G Watson-Crick proton to the O^6-atom. In each of the twelve different cases considered, the keto to enol conversion process is predicted to be thermodynamically favourable. These results thus predict that adduct-induced tautomeric shift of the N^1-G Watson-Crick proton could be a plausible mutagenic pathway for aromatic amine carcinogenesis.

In order to check upon the validity of this prediction, the normal and unusual tautomeric forms of free unmodified guanine were subjected to MNDO SCF MO calculation. The value of ΔH_{ke} for this simple system was -6.1 kcal/mole, clearly predicting the enol tautomer as stabler than the normal one. This prediction, however, is not in line with the known results of experiment, where the keto tautomer is invaraibly the stabler one at normal temperature and *p*H, whether as a free base, or nucleoside, or nucleotide or even as present in double-helical DNA. As such, the MNDO methodology is not found to be suitable for these tautomer

stability calculations. The predicted "stability" of the enol form over the keto is simply an artifact of the MNDO method, and the spurious results concerning guanine may be held in equal suspicion with regard to all the neutral carcinogen-DNA adducts as well. Thus, keto-enol tautomerism may be ruled out as a probable mutagenic or carcinogenic pathway for aromatic amine carcinogenesis.

V.4 Structures of Carcinogen-DNA Adducts

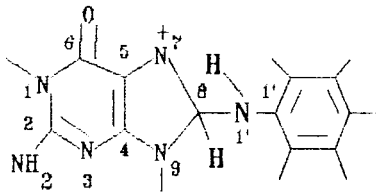
Previous experimental and theoretical studies have been given in the preceding chapter. This Section focusses on :

(1) Comparison of structure between the C^{8}-G and N^{2}-G adducts

(2) Effects of deprotonation upon structure of these adducts

V.4.1 Theoretical indices

Here, the atoms of the base are numbered without a dash ('), *eg* the N^{2}-atom of guanine is simply designated as N^{2}. The atoms of the carcinogen moiety, however, are numbered with a dash, eg. the imino nitrogen of the arylnitrenium moiety is numbered as $N^{1'}$.



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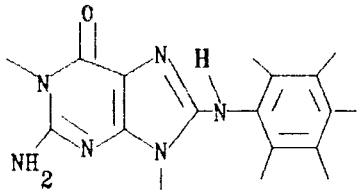

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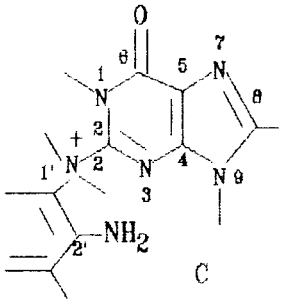

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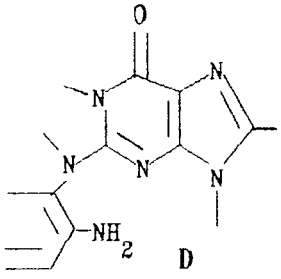


D

Fig.V.3: Atomic numbering and structures for cationic and neutral adducts around the binding sites. [A]: C^{8}-G cationic adduct; [B]: C^{8}-G neutral adduct; [C]: N^{2}-G cationic (II) adduct; [D]: N^{2}-G neutral (II) adduct.

The details concerning the adducts, and around the binding site in particulars, were gauged by three parameters (see Fig. V.3) :

(1) The bond length R_{cn} or R_{nc} between the base-site and the binding atom of the carcinogen moiety. The index R_{cn} refers to the C^8-G adducts, with binding between the C^8-carbon of guanine and the imino nitrogen of the nitrenium moiety; the index R_{nc} refers to the N^2-G adducts, with the bond between the N^2-nitrogen of guanine and the reactive carbon $C^{2'}$ conjugated to the imino nitrogen of the nitrenium moiety.

(2) the bond angle θ at the site of adduct formation. For the C^8-G adducts, this angle is defined as that encompassing the C^8-G, the imino nitrogen, and the carbon $C^{1'}$ bonded to the imino group of the nitrenium moiety. For the N^2-G adducts, θ represents the angle involving the C^2-G carbon, the N^2-G nitrogen of guanine, and the reactive carbon $C^{2'}$ of the nitrenium moiety attached.

(3) the torsional angle ϕ around at the site of adduct formation. For both C^8-G and N^2-G neutral adducts, this is defined as ϕ, the angle between the planes of the base moiety and the arylnitrenium moiety, measured by rotation around the bond formed between carcinogen and base. This dihedral for the C^8-G adducts is defined by the atoms $C^{1'}$-$N^{1'}$-C^8-N^9, and for the N^2-G adducts by the atoms $C^{1'}$-$C^{2'}$-N^2-C^2.

For comparison between cationic and neutral adducts, the dihedral ϕ was taken as the main parameter of interest. Changes

Table V.9: Geometry parameters for neutral carcinogen-DNA adducts*

	C8-G				N2-G		
species	R_{cn}	θ	ϕ	species	R_{nc}	θ	ϕ
2NA	1.41	117.8	146.3	2NA	1.44	123.1	132.7
4AB	1.42	115.8	121.9	4AB	1.44	121.3	134.6
BZ	1.42	115.9	112.6	BZ	1.44	121.4	126.2
2AF	1.41	117.8	148.5	2AF	1.44	119.7	115.8
2AAF	1.40	116.3	109.9	2AAF	1.44	121.3	126.9
MAB	1.42	117.1	132.3	MAB	1.44	120.1	127.7

Table V.10 : Comparison of structure for cationic and neutral adducts*

C^8-G	ϕ_{cat}	ϕ_{neu}	N^2-G	ϕ_{cat}	ϕ_{neu}
2NA	127.6	146.3	2NA	83.3	132.7
4AB	100.5	121.9	4AB	84.0	134.6
BZ	94.7	112.6	BZ	90.9	126.2
2AF	137.1	148.5	2AF	120.4	115.8
2AAF	125.1	109.9	2AAF	77.4	126.9
MAB	90.9	132.3	MAB	98.4	127.7

* R in Å
θ and ϕ in degrees

in ϕ would be expected due to deprotonation of the cationic adducts.

V.4.2 Results and discussion

Table V.9 presents the given parameters *R*, θ and ϕ for the optimized geometry of the neutral C^8-G and N^2-G adducts. It can be noted that the carcinogen-base bond formed is shorter (around 1.41 A) for the C^8-G adducts than the N^2-G adducts (around 1.44 A), which fits in well with the larger enthalpy of adduct formation for the former than the latter species (see Chapter Four).

For the C^8-adducts, the carcinogen moiety is not in the plane of the base, with varying ϕ values from 110^o to 146^o, which, when seen from the smaller values of the supplementary angle, comes to about 33^o to 70^o, this latter value being for the 2AAF adduct. A value of about 80^o was given for the interplanar dihedral angle forthe 2AAF adduct by minimised energy conformations at the molecular mechanics level (Hingerty & Broyde 1982), while experimental results indicated near perpendicularity. These three independent sources thus provide some degree of concurrence in their results for the 2AAF adduct. Since the carcinogen moiety for the C^8-G adducts protrudes into the major groove of the DNA helix (where steric interaction around is minimal), these MNDO conformational results for the solitary base may well hold for the

situation in the double helix itself. For the N^2-adducts too, the nitrenium moiety is not in the Watson-Crick H-bonding plane for all the cases studied (torsional angle varying from 116^o to 134^o). Here, the carcinogen moiety is not in the hydrogen-bonding region, but in the minor groove. As such, these simple solitary base results may not fully apply to the double-helical situation.

Table V.10 furnishes a comparison between the geometries of cationic and neutral adducts. The results show that for the C^8-G adducts, the dihedral ϕ is 91^o to 137^o for the cationic adducts, which then becomes 110^o to 149^o for the neutral ones, indicating for most cases a swing of the carcinogen-DNA bond more towards the plane of the base after C^8-deprotonation occurs. Similarly, the dihedral ϕ for the cationic N^2-G adducts has the range 77^o to 120^o, while the neutral values become 116^o to 133^o, indicating a similar turn of the carcinogen-DNA bond closer towards the guanine plane, but not to the extent of the C^8-G adducts. In all these cases, the picture will be more apparent if the appropriate values of the supplementary angles are taken where necessary. These conformational changes may be chiefly attributed to the change from sp^3 to sp^2 hybridization of the C^8-G and N^2-G atoms during deprotonation of the C^8-G and N^2-G adducts respectively. We may assume from the trends predicted by these MNDO calculations that the degree of change of hybridisation will be more obvious for the C^8-G adducts than the N^2-G adducts.

V.4 Conclusions

The following rationalisations, predictions and generalisations arise out of the content of work in this Chapter :

1. C^8-Proton lability for the C^8-G cationic adducts is chiefly due to soft contributions, whereas for N^2-G cationic adducts both hard and soft factors contribute to N^2-proton lability.

2. Thermodynamic indices for proton acidity predict the C^8-proton for C^8-G adducts and the N^2-proton for N^2-G adducts as the most acidic respectively in representative cases, as compared to the lower acidity predicted for Watson-Crick (N^1-G) proton.

3. Parameters for geometry around site of adduct formation reveal *(a)* stronger carcinogen-base bond strength for C^8-G adducts over N^2-G adducts; *(b)* conformational changes upon deprotonation due to change in hybridization of bonded atoms.

Chapter VI

CHAPTER VI

LOSS OF BASE OR CARCINOGEN MOIETIES FROM CARCINOGEN-MODIFIED NUCLEOSIDES AND BASES

VI.1 DNA Changes Induced by Bulky Carcinogens

VI.1.1 Mutations induced by bulky carcinogens

Polycyclic aromatic amines, together with polycyclic aromatic hydrocarbons and aflatoxins, comprise a class of carcinogens which may be generally termed as "bulky", being of high molecular weight. Gene mutations induced by bulky carcinogens and mutagens appear to operate in a manner somewhat different from those induced by simple alkylating agents or N-nitroso compounds. Several mechanisms have been proposed to rationalize the mutations induced by bulky carcinogens which can be categorised into the following (Loechler 1989, 1990) :

1. The adduct itself may be miscoding because the DNA polymerase recognises the modified base inaccurately
2. The adduct is proposed to be noncoding, implying that the DNA polymerase is unable to read the modified base
3. Indirect mechanisms where the adduct forms another mutagenic lesion, *eg.*, an apurinic site (AP site). In cells these sites are produced during the repair process of abnormal or modified bases.

The alterations in the base are specifically recognized and removed by highly specialized N-glycosylases (Sakumi & Sekiguchi 1990), generating these sites as intermediates in the repair system. In the context of this work, it is the generation of apurinic sites which emerges as of direct relevance for point mutation and carcinogenesis by aromatic amines.

A variety of biochemical and physical studies have been done to propose a specific model for the 3-dimensional conformation of nucleic acids modified by acetylaminofluorene or AAF (Fink *et al* 1970; Fujimura *et al* 1972; Grunberger & Weinstein 1971). Binding of AAF at the C^8 position of purines results in a large distortion of the DNA helix described as the "*base-displacement*" or the "*insertion-denaturation*" model (Grunberger & Weinstein 1976; Jeffrey *et al* 1980).

The first feature of this model (Nelson 1971) is that the attachment of the AAF residue to the C^8 position of G is

associated with a change in glycosyl conformation from *anti* to *syn*. The second major feature of this model is that there is a stacking interaction between AAF and a base adjacent to the substituted G residue. In addition, the planar fluorene ring system is inserted into the helix occupying the former position of the displaced guanine residue. It is also evident that the G residue displaced by AAF in the double helix cannot base-pair with the C residue of the complementary strand, and during the process of replication or transcription, no base-pairing at this position could occur (Levine *et al* 1974). In the context of mutagenesis and cancer, the *anti* to *syn* rotation associated with these modified nucleosides could prove capable of engendering point mutations of the base-transversional variety, specifically, the G to C transversion for this case. The effect of glycosyl rotation on mutagenesis has been studied (Venkateswarlu 1995) for C^8-oxidised purines.

The mutagenicities of different carcinogenic aromatic amines were determined in *Salmonella typhimurium* of different strains. Three broad classes of mutagenic activity were found. Interpretation of the results was as follows. The effect of class A (base-substitution without induction of error-prone repair) was produced by small aromatic amines (*eg.* 2NA) attached to extranuclear heteroatoms in DNA bases. That of class B (involving considerable induction of error-prone repair accompanied by a

lower level of frame-shifting) was caused by large aromatic group attached to extranuclear heteroatoms (*eg* 4AB) or by arylamines attached to C^8 of guanine. Class C (with a high level of frame shifting and some induction of error prone repair) was caused by arylamides attached to C^8- of guanine (Scribner *et al* 1979). Thus it is evident that the degree of bulk does seem to influence the type or class of mutations induced.

It was proposed that the GC to CG transversional mutation might be induced by *anti* to *syn* rotation of the glycosidic bond as induced by the carcinogen moiety of the C^8-G adducts of aromatic amines (Kadlubar 1980). Aromatic amine-adducts have also been proposed to be non-informational based upon *in vitro* investigations (Strauss *et al* 1982), and this could be the case for bulky adducts in general, *eg* for polycyclic aromatic hydrocarbons or aflatoxins. The possible reason for this is the excision of the modified nucleoside leaving a non-informational abasic site, as discussed below.

AP (apurinic/apyrimidinic) sites in DNA are generated by cleavage of the N-glycosyl linkage between the base and its deoxyribose moiety. These sites, which are known to be mutagenic (Loeb & Preston 1986), were proposed as a potential common intermediate in the induction of mutations by a variety of adducts (Loeb 1985). They are also intermediates in the biological repair of most base damage in DNA, being produced by specific

glycosylases that remove the abnormal or modified bases (Lindahl 1982; Weiss & Grossman 1987; Tchou & Grollman 1993). Artificial nucleases that recognize and cleave apurinic sites in DNA with great selectivity and efficiency have been prepared (Lhomme *et al* 1993). Substitution at the C^8-site of purine nucleosides modifies the hydrolytic stability of the nucleoside (Lhomme *et al* 1994), which can lead to depurination. How this could be of relevance for point mutagenesis, oncogene activation and carcinogenesis will be discussed in the coming sections of this Chapter.

VI.1.2 Proto-oncogene activation by carcinogens

The involvement of oncogenes in the induction of cancer is a well established proposal (Biswas 1989). An important mechanism discovered for the conversion of the proto-oncogene (latent) to its active form is that of point mutation at specific sites so as convert it to its carcinogenically active form (Balmain & Brown 1988). Oncogene activation by point mutation has been well documented for the *ras* family oncogenes (Rasheed *et al* 1983; Tabin *et al* 1982; Varmus 1984). The *neu* oncogenes in rat neuroblastomas have also been found to be activated by a point mutational mechanism (Bragmann *et al* 1986; Bragmann & Weinberg 1988). The critical mutations in the transforming *ras* genes have been found to occur at the 12th, 59th or 61st codons only, and all involve

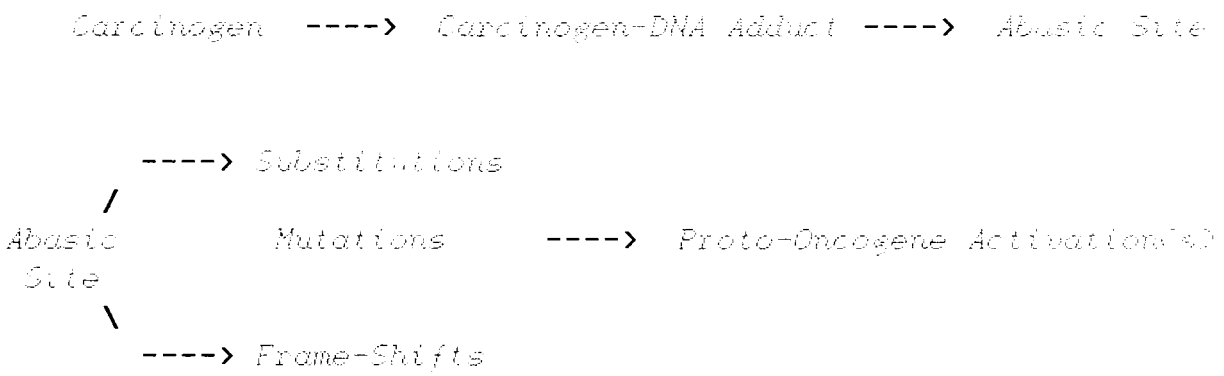
just single base substitutions, so that the protein product of the gene is changed in amino acid sequence at just one point only.

The base substitutions characterised include the G to A and the A to G transitions, as well as transversions like G to T, G to C, C to A, and A to T. For changes in the 12th codon of the oncogene, the amino acid glycine gets substituted by arginine, valine, glutamine, serine, cysteine, lysine and aspartine. Base substitutions in the 59th codon result in the replacement of alanine by threonine, while those in the 61st codon involve replacement of glutamic acid or histidine by leucine, lysine or arginine. Such single amino acid replacement in point mutation could have drastic effect on the conformational structure of the gene product with consequent effect upon biological activity.

It has been demonstrated how such seemingly small changes can have very significant effects on the biological activity of the *ras* oncogene product, with consequences for the cancerous development of the cell (De Vos 1988). Theoretical work has been done on the conformation of polypeptides (Pincus *et al* 1983) to establish that even single amino acid replacements can have drastic effects upon the secondary structure of polypeptide chains. NMR studies have been done for a DNA sequence from a *ras* protooncogene modified by 4-aminobiphenyl (Beland 1992).

It will be seen in the course of this Chapter that point mutational changes may be linked with known facts about the

biochemistry of carcinogen-DNA adducts formed by aromatic amines. The vital link here may well be supplied by the possibilities open for depurination of these adducts, leading to an abasic site which is a potential generator of non-specific base-substitutional mutations, as well as possibly single frame-shifts. The pathway for carcinogenesis may then proceed as follows :



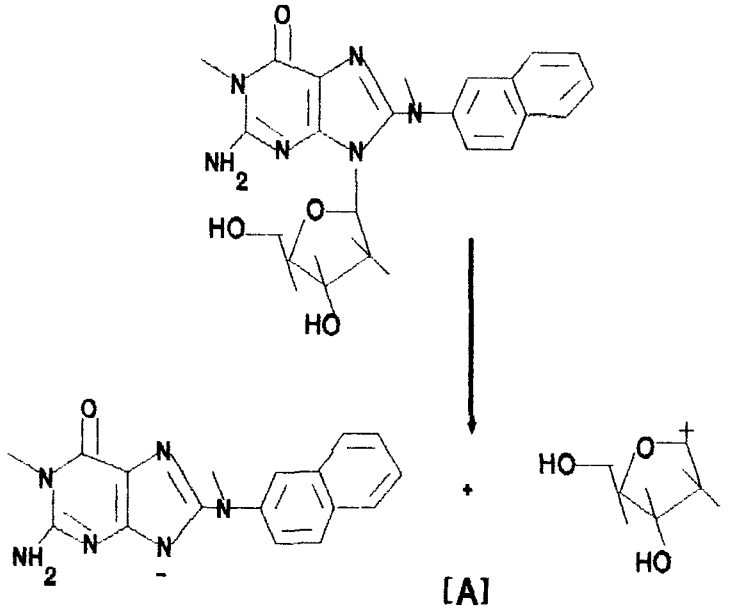
VI.2 Deglycosylation of Carcinogen-DNA Adducts

Deglycosylation refers to cleavage of the glycoside bond between a nucleic acid base and the pentose sugar moiety to which it is attached. This reaction may be also called "*depurination*" or "*depyrimidination*" depending upon whether the the base lost is a purine or pyrimidine. Glycosidic hydrolysis of intrastrand nucleotides yields AP sites, which have been characterized

(Manoharan *et al* 1988). Due to the relative instability of the N-glycosidic bond, such baseless sites in DNA may occur spontaneously with a relatively high frequency (Lindahl & Nyberg 1972). Depurination is much easier than depyrimidination since protonation to N^7 of guanine and to N^3 of adenine labilizes the glycosidic bond. Depurination is one of the most frequent forms of chemical damage to DNA (Kunkel *et al* 1983). It has been reported that *T4* DNA *polymerase*, DNA *polymerase I*, DNA *polymerase alpha* all stop at AP sites (Sagher & Strauss 1983). Hydrolysis of the glycosidic bond is accelerated by chemical alteration of bases with alkylating agents (Singer & Grunberger 1983) or through ionizing radiation (Teoule 1987). In certain alkyl nucleosides, it is connected to mechanisms for glycosylase repair. In the context of aromatic amine carcinogenesis, depurination assumes significance as a mechanism for generating potential mutagenic situations through an AP (apurinic) site, as just discussed earlier.

VI.2.1 Theoretical approach

Using the MNDO optimised geometry and wave-functions, the depurination reaction was modelled for the C^8-G and N^2-G adducts of deoxyguanosine with the 2-naphthylnitrenium ion by laying recourse to the following indices for deglycosylation facility :



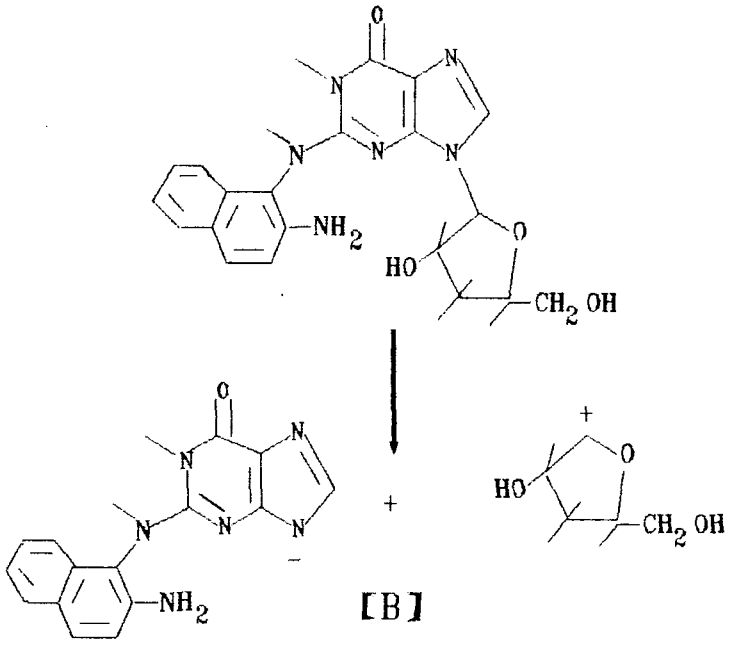


Fig. VI.1: The depurination reaction for the neutral forms of the 2NA adducts. [A]: The C^{8}-guanosine adduct; [B]: N^{2}-guanosine adducts.

These are considered along with pertinent indices for the facility of removal of the arylnitrenium moiety as well, in an effort to compare these two processes for the nucleoside adducts :

1. Deglycosylation enthalpies : In order to gauge relative strengths of the glycoside bond, the energy of bond dissociation was calculated in terms of the immediate products of bond breakage (without consideration of possible rearrangement to stabler products). Such an approach (in terms of immediate products of bond cleavage) could possibly be of some kinetic significance, and yield equable comparisons of bond strength accordingly. This use of the immediate products of bond cleavage for studying bond strength is thus useful, even though the indices are all simple reaction enthalpy changes without recourse to a formal study of the transition states involved. This approach may be compared with that of Ford and Scribner (1990), who suggested that the enthalpies of dissociation of alkanediazonium ions could be directly related to the activation energies of alkanediazonium ion attack on nucleophiles with specific relevance for the level of S_N1 or S_N2 character in the transition state for alkylation.

Fig. VI.1 portrays the depurination reaction for the neutral forms of the 2NA adducts at the C^8-G and N^2-G positions, where the enthalpy quantity is given as ΔH_{dg}. These reactions are considered in bare gas phase giving a pentose C-cation (as in Fig.

VI.1), as well as assisted by a water molecule (ΔH^{0}_{dg}), yielding an O-protonated pentose.

The corresponding bare gas phase enthalpy index for the reaction of loss or removal of the nitrenium moiety is ΔH_{dr} (Fig. VI.2 portrays the corresponding reactions for C^8-G and N^2-G neutral adducts).

2. Bond indices : These refer to strength of the concerned bond cleaved, *viz.* the N^9-$C^{1'}$ bond (R_{gb}, W_{gb}) for deglycosylation, and the C^8-$N^{1'}$ and N^2-$C^{2'}$ bonds (R_{nb}, W_{nb}) for loss of the arylnitrenium group, where the R and the W indices refer to bond length in angstrom and the Wiberg bond index respectively.

3. Relevant Mulliken charges : Facility of depurination was also gauged in terms of point charges (Q) on different relevant atoms such as the N^9-G (Q_{N9}), C_1'-dR ($Q_{C1'}$) atoms which come into play here in terms of the flow of the electron pair during depurination. Q_a is the point charge on the atom of arylnitrenium moiety (the amine nitrogen for C^8-adducts and the attacking carbon of the carcinogen moiety for N^2-adducts) and Q_b is the same on base sites (C^8- and N^2-) for the representative adducts in the case of arylnitrenium ion loss from the adducts with deoxyguanosine.

VI.2.2 Results and discussion

Tables VI.1 and VI.2 present the MNDO indices relating to deglycosylation facility for the C^8-G and N^2-G adducts

Table VI.1 : Indices for Deglycosylation process

species	ΔH_{dg}^{*}	$\Delta H_{dg}^{\omega *}$	R_{gb}	W_{gb}^{**}	Q_{N9}^{**}	$Q_{C1'}^{**}$
dG	186.1	192.8	1.48	0.87	-0.266	0.305
2NA-C^8-dG	167.8	174.6	1.48	0.86	-0.237	0.314
2NA-N^2-dG	192.8	199.6	1.47	0.87	-0.269	0.308

Table VI.2 : Indices for Arylnitrenium ion loss form deoxyguanosine-2NA Adducts

species	ΔH_{ar}^{*}	R_{nb}	W_{nb}^{**}	Q_b^{**}	Q_a^{**}
2NA-C^8-dG	184.0	1.42	0.99	0.144	-0.209
2NA-N^2-dG	193.3	1.43	0.96	-0.264	0.044

Table VI.3 : Enthalpy data for loss of arylnitrenium moiety for neutral adducts

C^8-adducts	ΔH_{dn}^{*}	N^2-adducts	ΔH_{dn}^{*}
2NA	192.8	2NA	185.8
4AB	195.5	4AB	194.8
BZ	196.6	BZ	197.4
2AF	187.3	2AF	190.9
2AAF	193.6	2AAF	191.7
MAB	203.8	MAB	203.9

* values in kcal/mole

** values in atomic units

R in Å

respectively of deoxyguanosine with 2-naphthylamine (2NA), the deoxynucleoside being taken in its neutral form .

The glycoside bond strength has been compared between the two adducts, as well as with normal (free) deoxyguanosine. The bond length, bond strength and bond dissociation enthalpy indices (ΔH_{dg}) all go to predict that the presence of the carcinogen moiety at the C^8-G site serves to weaken the glycoside linkage, making the adduct more prone towards depurination than the normal deoxyguanosine or the N^2-modified adduct. The glycoside bond dissociation enthalpies ΔH_{dg} in kcal/mole are as follows : deoxyguanosine (186.1); N^2-G adduct (192.8); C^8-G adduct (167.8). The other bond length, bond strength and bond order indices also provide a similar trend. The N^2-G adduct thus appears to be associated with a minimal tendency towards depurination, which is apparently even lower than that of unmodified deoxyguanosine. The predicted susceptibility of the C^8-G adduct to depurination is, as noted earlier, well substantiated by the experimental findings of Lhomme *et al* (1994) regarding C^8-modified guanine systems.

On the basis of these findings, it becomes possible to predict that, insofar as point mutagenesis *via* abasic sites are concerned, the C^8-G adducts would appear to have more relevance than the N^2-G adducts for the point mutagenesis and carcinogenesis processes. This is because depurination is predicted to be more facile for the former, so that C^8-G adduct formation would lead more easily

to abasic site creation than N^2-G adduct formation. These predictions are so far purely on the basis of theory alone, and require the results of experiment to bear them out. There is as yet no information on relative susceptibilities of these various carcinogen-DNA adducts towards depurination, nor any findings on their roles for non-specific base-substitutional mutagenesis or frame-shift mutagenesis. All these are potential areas of much interest with regard to elucidating the precise molecular basis for carcinogenesis and mutagenesis by aromatic amines. Sufficient to say for the moment that the depurination facility predicted here for the C^8-G adducts would not require much experimental infrastructure to substantiate; to go on from there to the actual molecular mechanisms for point mutagenesis would not be a very long step further in the right direction for molecular biologists to take.

It would also be a corollary inference that the C^8-G adducts would be more susceptible to glycosylase repair (through glycoside bond cleavage) than the N^2-G adducts. Such an inference takes it cue from the initial studies of Lyngdoh (1993) which linked repair mechanisms to chemical structure and reactivity of alkylated nucleosides. Since here again there exists no experimental information on glycosylase repair of carcinogen-DNA adducts for aromatic amines, we can only suppose that if glycosylase activity does indeed operate for these kind of lesions, then the C^8-G

adducts would be more amenable to this sort of repair than the N^2-G adducts. It is to be noted here that long patch repair is not connected with this glycosylase-mediated excision repair, and these studies have no relevance for long patch repair.

A comparison is now made between loss of the modified purine moiety and loss of the arylnitrenium moiety by itself, in analogy to the deglycosylation and dealkylation reactions and repair modes for alkylated nucleosides. Comparison of the relevant bond length and bond strength indices, as well as of the bond dissociation enthalpy indices (ΔH_{dg} and ΔH_{ar}) indicate that the deglycosylation process would be definitely more facile than loss of arylnitrenium group for the C^8-G adduct. The ΔH_{dg} index for depurination is calculated to be 167.8 kcal/mole, while the ΔH_{ar} index for loss of arylnitrenium group is appreciably higher, being 184 kcal/mole. The arylnitrenium moiety is thus predicted to bind much more strongly to the base than the sugar does.

For the case of the N^2-G adduct, the sugar and the carcinogen moieties appear to both bind to the base with about the same strength, as seen by the ΔH_{dg} value of 192.8 kcal/mole as compared to the ΔH_{ar} value of 193.3 kcal/mole.

The relative strong binding of the arylnitrenium group to the base is probably attributable to resonance interactions between the π clouds of the carcinogen and base moieties. This binding is predicted to be stronger than that of alkyl groups to various

DNA base sites, as seen from comparison made with values of alkyl group labilities calculated from the work of Lyngdoh (1994) and Venkateswarlu (1995). While alkyl groups bound to exocyclic oxygens in DNA bases are subject to facile spontaneous and enzymatic removal, it is doubtful whether this would be the case for the more strongly arynitrenium groups. The chemical removal of arylnitrenium moieties from DNA bound sites would be difficult compared to alkyl groups, while their enzymatic removal in analogy to alkyltransferase repair remains highly unlikely. The simple loss of the arylnitrenium moiety from carcinogen-base adducts is dealt with further in the next section.

VI.3 Loss of Arylnitrenium Moiety

In the case of certain alkylated DNA nucleosides, it has been noted that removal of the alkylating group alone (spontaneously or enzymatically) restores the original base/nucleoside (Lyngdoh 1993; Venkateswarlu 1995). Such a process has been shown to be of relevance, not so much for mutagenesis, but rather for repair of the DNA lesion. Here, the repair mechanism simply consists of removal of the alkyl group by transfer to a cysteine residue in the repair enzyme.

This section examines the possibility that this process might operate for DNA adducts formed by aromatic amines as well. In

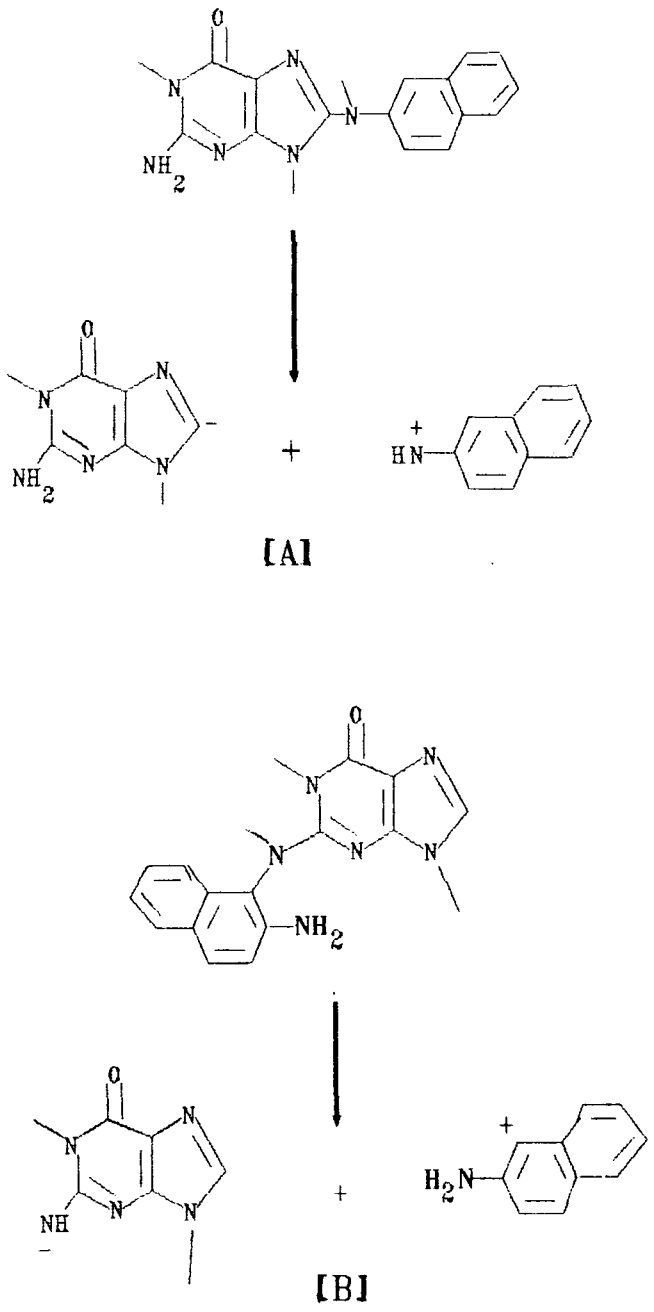


Fig. VI.2: The loss of arylnitrenium moiety in gas phase for 2NA neutral adducts. [A]: the C^8-G adduct; [B]: the N^2-G adduct.

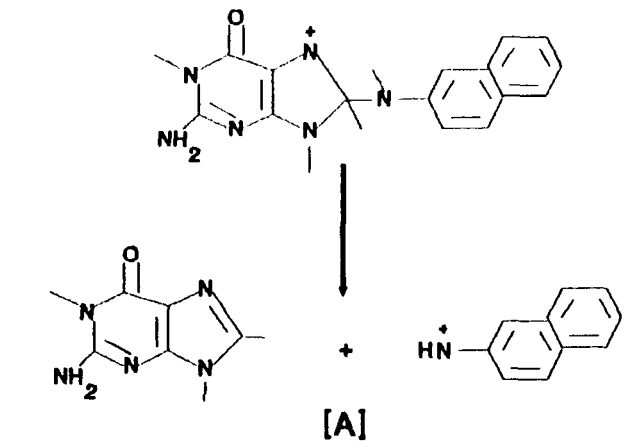
this case, the corresponding reaction would be loss of the arylnitrenium moiety from the C^8-G or N^2-G adducts, regenerating the original guanine base.

VI.3.1 Methodology

Using the MNDO optimised geometries and SCF wave-functions, the adducts of 2NA, 4AB, BZ, 2AF, AAF and MAB at the C^8 and N^2 sites of guanine were studied in their cationic and neutral forms. The reaction of removal of the arylnitrenium moiety was studied using the following physicochemical indices for feasibility of the process, all of which pertain to strength of the bond between the carcinogen moiety and the DNA base :

1. Enthalpy of the formal reaction process : Here, removal of the arylnitrenium moiety is here modelled by invoking the formal reaction products formed immediately upon bond dissociation without regard to rearrangement to a stabler species, which approach has been implemented with a view to mimicking the kinetic aspects of the process, where bond stability is the factor of prime importance. The rigorous approach of modelling transition states for each and every one of the 24 different cases involved here would have been much too compute-intensive to implement here in consideration of the current facilities.

For the neutral adducts, the bond dissociation reactions in gas phase are represented as by the case for 2NA in Fig. VI.2, where



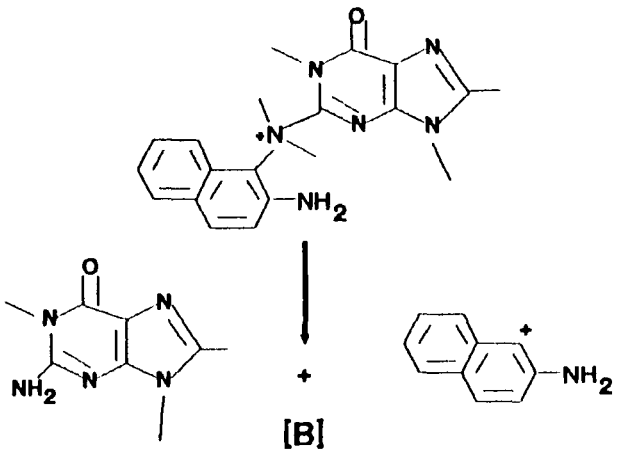


Fig. VI.3: The loss of arylnitrenium moiety in gas phase for 2NA cationic adducts. [A]: the C^{8}-G adduct; [B]: the N^{2}-G adduct.

both the C^8-G and the N^2-G adducts are included, the enthalpy being represented here as ΔH_{dn}. The only difference is that, here, the species considered is not the entire nucleoside, but only the modified base. The corresponding bond dissociation reactions in gas phase for cationic adducts are shown in Fig. VI.3 for the case of 2NA (both C^8-G and N^2-G adducts).

2. Bond strength indices : For the bond between the DNA binding site and the nitrenium ion, the indices of bond strength include (a) the bond length R_{ab}, and (b) the Wiberg bond order W_{ab} for the atoms "*a*" and "*b*" concerned for base and nitrenium moiety respectively.

The focus and aims of this study were :

(a) To study effect of pH and protonation on nitrenium group lability in the carcinogen-DNA adduct : Effect of pH of appropriate value is here modelled by use of cationic and neutral carcinogen-DNA adducts.

(b) To compare facility of nitrenium moiety removal between the C^8-G *and the* N^2-G *adducts*

Note that it has been deemed necessary to simply substitute the whole modified nucleoside with just the modified base alone here for this study of arylnitrenium removal, since calculation of optimised geometries for all 24 cases in their nucleoside form would have been computationally most demanding.

Table VI.4 : Bond indices for loss of arylnitrenium moiety for neutral adducts*

C^8-adducts	R_{cn}	W_{cn}	N^2-adducts	R_{nc}	W_{nc}
2NA	1.410	0.995	2NA	1.434	0.962
4AB	1.417	0.987	4AB	1.436	0.960
BZ	1.416	0.988	BZ	1.437	0.960
2AF	1.412	0.992	2AF	1.441	0.959
2AAF	1.408	0.998	2AAF	1.435	0.962
MAB	1.418	0.987	MAB	1.440	0.959

Table VI.5 : Bond indices for loss of arylnitrenium moiety for cationic adducts*

C^8-adducts	R_{cn}	W_{cn}	N^2-adducts	R_{nc}	W_{nc}
2NA	1.435	1.021	2NA	1.487	0.896
4AB	1.439	1.017	4AB	1.485	0.899
BZ	1.444	1.008	BZ	1.484	0.901
2AF	1.442	1.012	2AF	1.486	0.899
2AAF	1.430	1.030	2AAF	1.485	0.899
MAB	1.431	1.031	MAB	1.487	0.897

* R in Å
W in atomic unit

Tables VI.3 to VI.5 present the bond dissociation enthalpies and bond strength indices for loss of the arylnitrenium moiety from the neutral and cationic adducts (both C^8-G and N^2-G) - a total of 24 cases in all.

The bond dissociation enthalpy data in Table VI.3 for six C^8-G and six N^2-G adducts (all neutral) indicate that the loss of the arylnitrenium moiety would be thermodnamically about as feasible for the neutral C^8-G adducts as for the N^2-G adducts. The range of values for ΔH_{dn} (about 185 to 204 kcal/mole) corresponds well with the expected range for heterolytic fission of a strong covalent carbon-nitrogen bond. However, the bond indices of Table VI.4 for the neutral adducts predict that the arylnitrenium group is more labile for the N^2-G adducts than the C^8-G adducts, having a longer bond length R_{ab} value range, and a higher bond strength index W_{ab} range as well (R_{cn} and W_{cn} for C^8-G adducts are between the C^8-atom of guanine and nitrogen of arylnitrenium moiety; and R_{nc} and W_{nc} for N^2-G adducts are between N^2-atom of guanine and attacking carbon of arylnitrenium moiety).

The corresponding data for the cationic adducts is given in Table VI.5 . It is immediately evident upon inspection that loss of the arylnitrenium group is predicted to be much more facile for the cationic adducts than for the neutral ones, which is true for

each and every case studied. This inference is drawn from lengthening of the bond concerned and smaller values of the Wiberg bond index upon protonation. These findings have much in common with earlier studies (Lyngdoh 1994; Venkateswarlu 1995) on the effect of pH and protonation upon alkyl group labilities in alkyl DNA nucleosides and bases. However, owing to the paucity of experimental information on arylnitrenium group labilities in these systems or their susceptibility to repair, it is not possible to relate these theoretical predictions to established experimental data. It is possible, at the most, to predict that lowering of pH would be expected to weaken the bonding between carcinogen and DNA base in these adducts.

It is also further evident that, for each carcinogen studied, the cationic N^2-G adducts are marked by an appreciably greater lability of the arylnitrenium group than the C^8-G adducts. This inference is unanimously attested to by the bond length and bond strength indices for the 12 cationic adducts. It may be interpreted in terms of the comparatively less scope for resonance strengthening of the concerned bond in the case of the N^2-G adducts than in the case of the C^8-G adducts, the reason for which may be evident upon inspection of the structures. This disparency in arylnitrenium group labilities between C^8-G and N^2-G adducts is more obvious here for the cationic adducts (Table VI.5) than for the neutral ones (Tables VI.3 and VI.4).

VI.4 Conclusions

The following generalisations can be made out of the content of the work in this Chapter :

1. Depurination is predicted to be more feasible pathway for C^8-arylnitrenated guanine systems than for the corresponding N^2-G adducts, owing to which, abasic sites may be created more easily with the former than the latter.

2. As a consequence of (1) above, non-informational mutagenesis is more likely for C^8-G adducts than N^2-G adducts, with possible implications for the cancer process.

3. The arylnitrenium moiety appears to be more labile at the N^2-G site than at the C^8-G site, while transferase repair through removal of the carcinogen moiety alone seems unlikely for any kind of adduct.

4. The C^8-G adducts are predicted to be more prone towards N-glycosylase repair than the N^2-G adducts.

5. The effect of lowering pH to more acidic values would be to facilitiate removal of the arylnitrenium moiety from adducts.

List of Abbreviations

A	adenine
AO	atomic orbital
$ArNH_2$	aromatic amine
$ArNH^+$	aromatic nitrenium ion
B	base
C	cytosine
C-onc	cellular oncogene
DNA	deoxyribonucleic acid
G	guanine
HOMO	highest occupied molecular orbital
HSAB	hard-soft acid-base
LUMO	lowest unoccupied molecular orbital
MNDO	modified neglect of differential overlap
MO	molecular orbital
PaC	parent carcinogen
PrC	proximate carcinogen
SCF	self-consistent field
SOMO	singly occupied molecular orbital
T	thymine
UC	ultimate carcinogen
V-onc	viral oncogene

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