Mechanistic Models For Carcinogenic Activity, Potency Index, And Choice Of Target Organ For Carcinogenesis By N-Nitroso Compounds.

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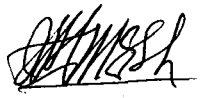

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PREFACE

Chapter One is an introduction to the field of chemical carcinogenesis in general, focussing on the molecular aspects of the phenomenon. It is laid down as a guiding principle of the work here that reduction of the complex biological phenomenon of carcinogenesis to precise molecular terms (whether from the results of experiment or hypothetically) is a pre-requisite to the tackling of the problem through the use of theoretical chemistry. The ultimate carcinogen theory and the somatic mutation theory are marked out as providing the key pivotal concepts around which such an investigative approach revolves.

Chapter Two is a review of the biochemical and molecular biological mechanisms for *N*-nitroso carcinogenesis, as deduced from the work of various groups of experimentalists. A precise sequence of molecular events is put forward as furnishing a viable molecular basis for the phenomenon. The successive stages consist of *(a)* administration and transport of the carcinogen to and within the living system, *(b)* metabolic or spontaneous conversion of the parent carcinogen to its electrophilic ultimate carcinogenic form, *(c)* approach and attack of the critical sites on DNA by the ultimate carcinogen, *(d)* induction of the relevant mutations at the critical sites on the proto-oncogenic sequence *(e)* transformation of the proto-oncogene to its carcinogenically active form *(f)* expression of the activated oncogene through the oncoprotein products it encodes

for, and *(g)* participation of the oncoproteins in the cellular and macro-molecular changes involved in the initiation and maintenance of the cancerous state. That each of these molecular events is of consequence for the macroscopic and phenomenological features of *N*-nitroso carcinogenesis is a key dictum for the approach of this thesis.

Chapter Three tackles one of the chief phenomenological features, *viz.* the presence or absence of carcinogenic activity in a particular *N*-nitroso compound. Viewing firstly from the role played by the ultimate carcinogen, a series of mechanistic criteria are proposed which could serve to establish whether or not carcinogenic activity would be present. These criteria are drawn from the mechanistic role of enzymatic or spontaneous chemical conversion of the parent carcinogen, and from the role played by deactivating hydrolysis or cyclisation of the ultimate carcinogen species. Some of these criteria are expressed in a quantitative form either empirically or from quantum chemical calculations (using the MOPAC package). Application of these criteria based upon the ultimate carcinogen concept serves to differentiate quite effectively between carcinogen and non-carcinogen for a large number of cases. Some cases which cannot be explained by the ultimate carcinogen concept are reserved for treatment using the second phase of the cancer process, *viz.* the role of modified DNA for carcinogenesis.

Chapter Four invokes the crucial role of modified DNA in the determination of presence/absence of carcinogenicity. Basing upon current knowledge available on the underlying mechanisms, a series of criteria are proposed which focus on the *O*-selectivity of DNA modification, the role of the Watson-Crick protons, and on the conformational role of the exocyclic *O*-alkyl group in certain *O*-alkylated DNA bases. The importance of *O*-alkylation has been established by *in vitro* and *in vivo* studies on the miscoding and mutagenic properties of several *O*-alkylated bases. Application of these criteria through quantification and molecular orbital analysis furnishes some effective rationalisations regarding the inactivity of certain alkylating esters (like methyl methanesulphonate) and the complete lack of carcinogenic activity in any *N*-nitroso compound containing a tertiary alkyl group structure at the α-carbon. Through joint application of the complete set of criteria drawn from the work of Chapter Three and Chapter Four, a good demarcation into active and inactive members is achieved among the class of some 332 *N*-nitroso compounds so far tested. The few cases not explained are given some hypothetical rationalisations which, however, require further work and thought. On the basis of this mechanistic model, a number of hitherto untested *N*-nitroso compounds are accordingly predicted as active or inactive, which results await experimental confirmation.

Chapter Five deals with the well-observed variations in cancer-inducing power or potency demonstrated among the class of active *N*-nitroso compounds. The dose-response data of Druckrey *et al.*,

(1967), when coupled with the quantifying scheme of Wishnok-Archer (1967), provide a large and internally consistent set of numerical data on relative carcinogenic potency to work upon. Focussing again only on rat carcinogenesis, a series of mechanistic factors influencing or modulating the parent carcinogenic potency are isolated. These include *(a)* the organ in which cancer occurs, *(b)* the expected concentration of the ultimate carcinogen generated, *(c)* the *O*-selectivity of DNA alkylation, *(d)* the effectiveness of DNA alkylation, and *(e)* the rate at which DNA repair occurs as a function of the alkylating group concerned. All the factors are quantified, either empirically or through theoretical chemical calculations, and are also considered together in an attempt to construct a numerical composite index for predicting carcinogenic potency.

Chapter Six concerns the application of mechanistic and physiological criteria to explain the remarkable organ-specific effects observed in *N*-nitroso carcinogenesis, consideration being limited now only to rats. The classification into groups according to the chief target organ for tumourigenesis is seen to follow remarkable structural lines. Symmetrical dialkylnitrosamines are liver carcinogens in rats, while asymmetrical dialkylnitrosamines are oesophagal carcinogens. Likewise, alkylnitrosoureas exhibit a systematic neurotropy of carcinogenic effect. It is also seen that direct-acting *N*-nitroso compounds can induce cancers and tumours around the site of application.

The mechanistic and physiological criteria drawn up to build a model for rationalising and predicting these organotropic effects takes into consideration *(a)* the route and site of administration of the parent carcinogen, *(b)* the *in vivo* stabilities of the compounds administered, *(c)* the various transport systems available in the host animal, *(d)* the organspecific location of the appropriate metabolising enzymes, and *(e)* the organspecific location of the appropriate DNA repair enzymes. On putting the results of previous experimental work together in this manner, together with some cogent assumptions, a coherent model is built up to explain and predict the choice of target organ for cancers and tumours for a wide range of cases. The model is applied to rationalise existing observations, as well as to also furnish predictions for a number of cases not yet covered by experiment.

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INTRODUCTION

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I.1 The Phenomenon of Carcinogenesis

Some diseases are as old as man, perhaps even older. Cancer seems to have been almost a natural companion of man in the course of his earthly existence, having been part and parcel of human life at large ever since man appeared on this planet. Cancer is not confined to the species *Homo sapiens* alone, being capable of ravaging every kind of vertebrate. Basically, cancer is a cellular disorder characterised by the rapid and uncontrolled proliferation of cells which have been genetically altered in a specific manner. This gives rise to the growth and spread (metastasis) of tumours, which in principle may occur at any organ of the body. At the cytological level, apart from uncontrolled cell division, other key features include the breakdown of normal mechanism for growth, regulation and differentiation, marked changes in cell shape and motility, and a tendency towards the anaerobic mode of cellular respiration. Cancer, if unchecked, can lead to the death of the organism.

Indeed around 20 to 25% of human deaths are attributed to cancer-related causes.

A multistage process may be invoked to describe the onset and development of cancer, consisting of the fairly well-defined successive stages of *cancer initiation*, *promotion* & *tumour growth and progression*, and *metastasis*. Initiation involves the creation of a potential cancer cell following critical action of the *initiating agent*, whose cellular target is the nuclear DNA of the somatic cells. Initiated cells may pass through a latency period until induced to start multiplying by the *promoting agent*. This stage of tumour growth and promotion maintains the proliferative and undifferentiated state of the cell mass. Finally the tumour acquires the capacity for metastasis or invasive spread of cancer to various parts of the body. This is the malignant phase, when invasion of tissues leads to colonisation on secondary sites, the major factor in cancer fatalities.

Environmental causes of cancer may be attributed to:

(a) Physical factors, *eg.* electromagnetic or particulate radiation, heat or pressure

(b) Biological factors, *viz.* tumour viruses, and

(c) Chemical factors.

Upto 90% of human cancers today may be attributed to environmental chemical carcinogens. Epidemiological studies have also earmarked gamma radiation and nuclear fission products as important causes of cancer, eg. among the victims of the atomic bomb holocausts of World War II. Apart from the Epstein-Barr virus (concomitant with endemic malaria among African children)

and the human papilloma virus, tumour viruses have not in general been found to be operative in man, although well-known in various animal species ever since the discovery of the chicken sarcoma virus. The breakdown in immunological defences accompanying HIV infection is a secondary factor in the widespread occurrence of sarcomas among AIDS patients.

I.2 Salient Features of Chemical Carcinogenesis

The phenomenon of chemical carcinogenesis may be viewed from two perspectives, *viz.* the *macroscopic* and the *microscopic* (or molecular) aspects. The clinical, epidemiological and histopathological aspects pertain to the former, while the latter concerns the description of the cancer phenomenon at the molecular and mechanistic level. One of the chief objectives of theoretical chemists and molecular biologists is to explain the former in terms of the latter. A hierarchy of levels of understanding of the cancer process may thus be built up as follows:

(a) The *epidemiological* understanding of cancer, which is primarily statistical, being mainly directed towards *whole living populations.*

(b) The *clinical* understanding of cancer, which is chiefly orientated towards humans, covering diagnosis and treatment, being directed towards *individuals.*

(c) The *histopathological* understanding of cancer, which deals with the change in *organs* or *tissues* of cancer patients.

(d) The *cytological* understanding of cancer, which treats the alterations occuring in *cells* of affected tissues and organs.

(e) The *molecular biological* understanding of cancer, which concerns with the precise macromolecular and biomolecular interactions and the structural changes accompanying the process of carcinogenesis.

(f) The *toxicological* understanding of cancer, which deals with the interactions between the carcinogen and components of the living system.

(g) The *bioorganic* understanding of cancer, arising from the conjunction of biochemistry and organic chemistry, which analyses the mechanism of cancer in terms of molecular structure, reactivity and interactions.

(h) The *quantum chemical* understanding of cancer, arising from the application of the quantum theory of molecular structure and interaction to the molecular phenomena of *(g)* above, which thus studies carcinogenesis at the most fundamental and irreducible level possible.

At the macroscopic and phenomenological level of understanding, the following *salient features* of the carcinogenesis phenomenon emerge as significant and relevant, seen here from the viewpoint of chemical carcinogenesis in test animals, and which form the pivotal points for the studies embodied in this dissertation :

(a) The presence or absence of *carcinogenic activity* in a particular chemical tested on a particular animal species.

(b) The abundance of tumour yield, leading to the assignment of *relative carcinogenic potency* among a series of chemical carcinogens.

(c) The choice of *target organ(s)* for tumourigenesis in the test animal.

The above salient features are dealt with in detail as below:

(a) Presence or Absence of Carcinogenicity

Animal testing for carcinogenicity is still the only definitive screening test for determining whether a given chemical is a carcinogen or not. Besides being expensive and time-consuming, it also has to contend with the problem of extrapolation to human carcinogenicity. The *Survey of Chemicals Tested for Carcinogenicity (1951-1988)*, as brought out by the National Institutes of Health (United States Government) in a series of volumes, is the most thorough and exhaustive compilation of data on such testings to date, of much value for all classes of researchers.

Generally speaking, chemical carcinogenicity runs in families, where a good proportion of the members of a class of structurally related or homologous compounds exhibit carcinogenic activity, *eg.* among polycyclic aromatic hydrocarbons, polycyclic aromatic amines, aflatoxins, *N*-nitroso compounds and alkylating agents. Although there is a high risk factor present in such families, the fact that some members are not carcinogenic poses the need for individual screening for each member. Chapters III and IV attempt to furnish some simple theoretical alternatives to long-term animal tests for screening for carcinogenicity in *N*-nitroso compounds.

The concept of presence or absence of carcinogenicity is not such a simple or clearcut one. This is due firstly to the observation that a particular chemical may be a carcinogen in one species of animal and not in another. Secondly, there also appear to be some borderline cases, probably arising out of

insufficiency of dose administered or an inefficient dosage schedule. Lastly, some chemicals do not act as carcinogens in themselves, but only when co-administered with some other chemical- the phenomenon of co-carcinogenesis.

(b) Variations in Relative Carcinogenic Potency

Difficult as the concept of presence or absence of carcinogenic activity is to define, the problem of assigning relative carcinogenic potency within a series of chemicals requires even more careful thought and judiciousness. A number of methods have been devised to gauge relative carcinogenic potency. These include the Iball index for carcinogenic potency of polycyclic aromatic hydrocarbons (Iball, 1939), the Wishnok-Archer (1967) potency index for *N*-nitroso compounds, and the use of a varying number of plus signs (+) to indicate lower or higher potency (Andrews & Lijinsky, 1984).

Chapter V deals with the rationalisation and prediction of relative carcinogenic potency for *N*-nitroso compounds. The various mechanistic factors modulating potency are isolated, quantified theoretically, and correlated with the existing experimentally derived data on relative carcinogenic potency. The success or failure of the correlations observed may be used to indicate which mechanistic steps or factors could be of direct relevance to the carcinogenesis bio-pathway.

(c) Choice of Target Organ for Tumorigenesis

Administration of a carcinogen to animal does not induce tumours or cancers in each and every organ of the animal. To a

large extent, choice of target organ for tumourigenesis is confined to one or a few organs or organ systems. Factors of relevance to choice of target organ include administration route, dosage schedule, *in vivo* carcinogen stability and various biological factors dependent on the animal species and organs concerned. One of the most fascinating features of *N*-nitroso carcinogenesis is the highly specific choice of target organ for cancer induction, often independent of administration route in test animals. The various mechanistic and biological factors contributing to the choice of target organ are discussed in Chapter VI, where they are incorporated into a simple model for rationalisation and prediction of target organ for *N*-nitroso carcinogenesis.

I.3 General Mechanisms for Chemical Carcinogenesis

As will be evident from the title of this thesis, it is assumed as a starting point for this work that the molecular understanding of carcinogenesis is capable of furnishing much insight into the macroscopic aspects of this biological phenomenon. Reducing the cancer process to molecular terms, or expressing the phenomenon in molecular language, indeed lies at the heart of the quest pursued by molecular biologists, biochemists and theoretical chemists, who have applied their minds and skills towards tackling the phenomenon of carcinogenesis. It may be stated as a dictum that any attempt of a theoretical chemist to study cancer is subject to this reduction of the carcinogenesis phenomenon to precise molecular terms, whether based on experimental findings or simply hypothetically. The arsenal of study tools used by the

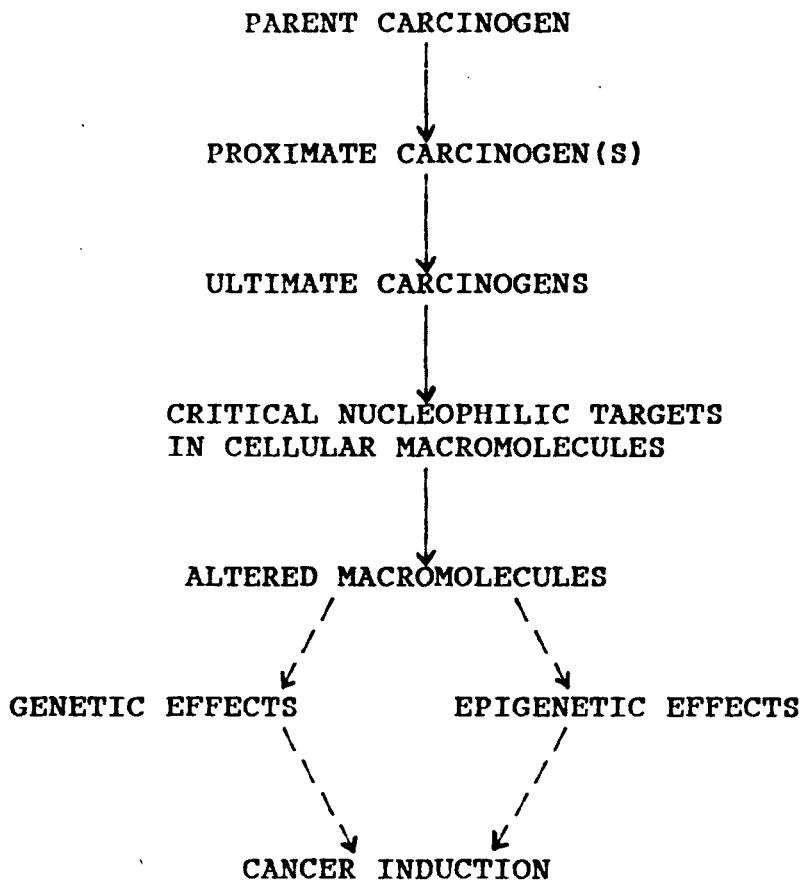
theoretical chemist, including the quantum theory of molecular structure and interaction and various empirical approaches, is of meaningful and practical utility for studying cancer-associated problems only when the phenomenon is expressed in *precise molecular language.*

This section deals with some general approaches to elucidation of the molecular mechanisms underlying chemical carcinogenesis. A survey of the various families of chemical carcinogens has demonstrated that, to a large extent, there exists a good deal of broad mechanistic commonality in the molecular basis of their carcinogenic action. This common approach to the mechanistic understanding of chemical carcinogenesis stems from two important concepts which have vastly increased modern man's understanding of the molecular basis of cancer, *viz.* the ***ultimate carcinogen theory*** and the ***somatic mutation theory***, each dealt with as follows :

(a) The Ultimate Carcinogen Theory

The ultimate carcinogen theory, first propounded by Miller and Miller (Miller, 1970; Miller & Miller, 1977), is now widely accepted as fact. This theory has introduced a major unifying dimension to our understanding of chemical carcinogenesis by proposing that the vast majority of (if not all) chemical carcinogens, being relatively inert in themselves, are converted *in vivo* to *reactive electrophilic species* either through spontaneous decomposition (Brundett, 1980; Lown *et al.*, 1978; Lown & Chauhan, 1981) or by enzymatic activation (Montesano & Magee, 1970; Druckrey, 1975; Dutton & Heath, 1956). These

Figure I.1: General sequence of events of the Ultimate Carcinogen Theory of chemical carcinogenesis.



reactive electrophilic species, being the actual agents that critically modify nuclear DNA and trigger off the cancer process, are termed as *ultimate carcinogens* (or "UC" for short). The initial compound so administered is known as the *parent* carcinogen and the intermediate reactive precursor is called the *proximate* carcinogen(s), while the reactive electrophilic species that generally modify the nuclear DNA is termed as the *ultimate* carcinogen.

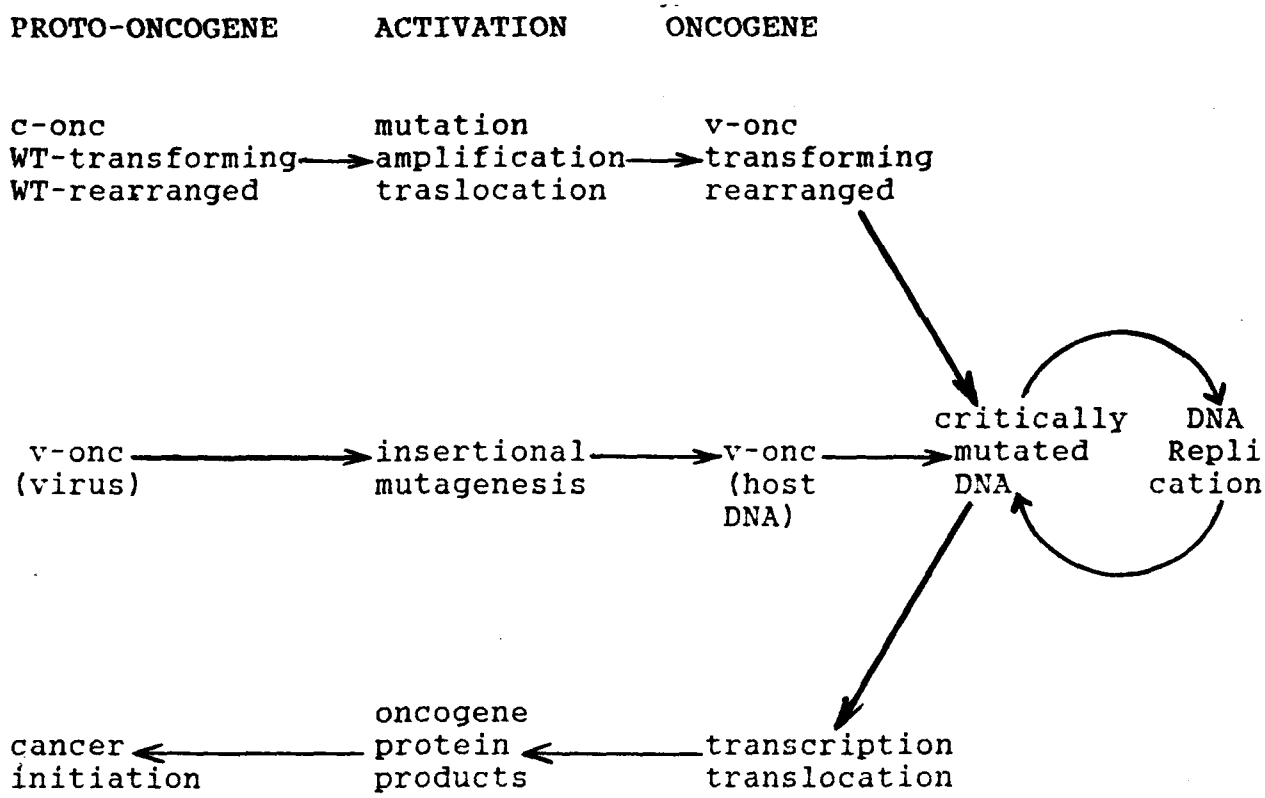
The general sequence of events as put forward by the ultimate carcinogen theory of chemical carcinogenesis is portrayed in the Figure I.1.

(b) The Somatic Mutation Theory

The somatic mutation theory of cancer, which was initially put forward in germinal form by Boveri (1914) and later worked upon or refined further by Lowdin (1977), Ts'O (1980) and Skipper (1983), proposes a genetic origin for cancer. The DNA being the prime genetic material, this theory postulates a key role played by the critically altered nuclear DNA of somatic cells for the induction and maintenance of the cancerous state (Strauss, 1981; Bishop, 1985). DNA is known to be definitely altered and modified by chemical or physical carcinogens. The ample evidence for the detection and the characterisation of modified DNA constituents chemically modified by carcinogens also points to this. Chapter IV treats this in greater detail.

The discovery of the existence and functioning of oncogenes, reviewed by Franks and Teich (1986), lends direct confirmation to this concept of somatic mutation, suggesting that modifications

Figure I.2: Mechanism of proto-oncogene activation and general concepts of the Somatic Mutation Theory.



only at the critical sites on the proto-oncogene would be of carcinogenic significance (Varmus, 1984). Thus, the mutation resulting from DNA modification may be simply a point mutation like base-substitution or frame-shifts, or involve actual breakage or translocation of a chromosome. Gene amplification serves as another mechanism for involvement of DNA in carcinogenesis. Such a transformed cell becomes capable of indefinite cell division independent of normal control mechanisms, thereby leading to tumour growth and development.

Fortunately for biochemists, molecular biologists and microbiologists, this key concept becomes easily accessible to testing because of the amount of experimental evidence related to modifications of DNA and even entire cell lines during carcinogenesis by radiation, by chemicals and by viruses. The somatic mutation concept is strengthened by the findings of molecular biology regarding cellular transforming or *c-onc* genes (Cooper, 1982), viral transforming or *v-onc* genes, transfection experiments (Newbold & Overell, 1983) with *v-onc* hybrids originating from viruses (Harris *et al.*, 1979; Shay *et al.*, 1981). Thus, the general consensus of opinion today is that most cancers have a genetic origin, as contrasted with the concept of the epigenetic origin of cancer. The various mechanisms involved in neoplastic transformation of DNA, *viz.* those of proto-oncogene activation, chromosomal translocation and gene amplification, as invoked by the general concepts of the somatic mutation theory are portrayed in Figure I.2.

I.4 Chemical Carcinogenesis by N-Nitroso Compounds

Carcinogenesis by N-nitroso compounds (NOC) and alkylating agents has evolved into a major field of cancer research in the nearly four decades that have elapsed since the discovery of the carcinogenicity of dimethylnitrosamine (Magee & Barnes, 1956). Since then, research on chemical carcinogenesis by NOC has mushroomed almost exponentially covering various aspects like analysis and occurrence, formation from precursors, basic chemistry, biological activity (carcinogenicity, mutagenicity, teratogenicity and toxicity) and biochemistry (Preussmann & Stewart, 1984). A number of illuminating monographs have come out surveying the phenomenon (Preussmann & Stewart 1984; Rao *et al.*, 1984; Bartsch *et al.*, 1982; 1987; Hiatt *et al.*, 1977; Searle, 1984). Some of the compelling reasons for this deep interest in the phenomenon are as follows:

(a) The potent carcinogenicity in test animals of most *N*-nitroso compounds tested so far.

(b) The remarkable organ-specific effects of *N*-nitroso compounds in test animals.

(c) The relevance of NOC for human cancer, as evinced from the occurrence of certain NOC in the human environment through pollutants, certain consumables, cosmetics (Fan *et al.*, 1977), pesticides and industrial chemicals (Druckrey & Preussmann, 1962; Enders *et al.*, 1964; Sakshaug *et al.*, 1965; Ohshima & Bartsch, 1981; Schmahl, 1980; Scanlan & Tannenbaum, 1982).

(d) The possibility of *in vivo* formation from precursor amines and nitrosating agents (Dubsky & Spritzmann, 1916; Ridd, 1961;

Druckrey *et al.*, 1963; Shank & Newberne, 1976; Singer & Lijinsky, 1976; Spiegelhalder *et al.*, 1979).

(e) The remarkable clarity with which the biochemistry and the molecular biology of *N*-nitroso related biological phenomena (including carcinogenesis) is known to modern science.

(f) The easy synthesis and availability of *N*-nitroso compounds

N-Nitroso compounds are classified broadly into *N-nitrosamines* and *N-nitrosamides*. The first broad class of *N*-nitrosamines consists of the dialkylnitrosamines (symmetrical & asymmetrical), the cyclic nitrosamines and dinitrosamines. These are generally chemically stable compounds, which require metabolic activation mediated by mixed function oxidases or cytochrome P_{450} systems which are also responsible for the metabolism of many drugs and xenobiotics (Magee & Vandekar, 1958; Brouwers & Emmelot, 1960).

The second broad class consist of the *N*-nitrosamides, which includes the subclasses of *N*-nitrosoureas, *N*-nitrosourethanes, *N*-nitroso-*N'*-nitroguanidines and *N*-nitrosamides proper. Most *N*-nitrosamines are *indirect-acting* because they require metabolic conversion in order to exert their carcinogenic effects. On the other hand, *N*-nitrosamides are chemically less stable compounds not requiring enzymatic bio-activation since they decompose spontaneously *in vivo* (with the exception of *N*-nitrosotrialkyl-ureas (Lijinsky & Taylor, 1975; Lijinsky *et al.*, 1980). As such they are termed as *direct-acting* carcinogens.

According to the new classification adopted here, dialkyl-nitrosamines may be classified as symmetrical or asymmetrical depending upon whether or not the two alkyl groups are identical.

Table I.1 Classification of *N*-nitroso compounds giving numbers and percentage of active and inactive member in each class.

Class	Total No.	No. +ve	No. -ve	% +ve	% -ve
A. N-NITROSAMINES	232	193	39	83.2	16.8
1. Symmetrical DRNA	55	44	11	80.0	20.0
(a) Unsubstituted	16	10	6	62.5	37.5
(b) C-oxidised	25	23	2	92.0	8.0
(c) C-substituted & others	14	11	3	78.6	21.4
2. Unsymmetrical DRNA	95	81	14	85.3	14.7
(a) Unsubstituted	51	46	5	90.2	9.8
(b) C-oxidised	32	25	7	78.1	21.9
(c) α-acyloxyalkyl[c]	6	5	1	83.3	16.7
(d) C-substituted & others	6	5	1	83.3	16.7
3. Cyclic nitrosamines	71	60	11	84.5	15.5
(a) Unsubstituted	15	15	0	100.0	0.0
(b) Ring C-oxidised	14	7	7	50.0	50.0
(c) Ring C-substituted	19	16	3	84.2	15.8
(d) Ring with hetero-atoms[a]	23	22	1	95.7	4.4
4. Dinitrosamines[b]	11	8	3	72.7	27.3
B. N-NITROSAMIDES	100	88	12	88.0	12.0
1. N-nitrosoalkylureas	54	50	4	92.6	7.4
(a) Monoalkylureas	33	29	4	87.9	12.1
(b) Dialkylureas	20	20	0	100.0	0.0
(c) Trialkylureas[d]	1	1	0	100.0	0.0
2. N-nitrosourethanes	17	17	0	100.0	0.0
3. N-nitroso-N'-nitro guanidines	11	6	5	54.6	45.5
4. N-nitrosamides proper	18	15	3	83.3	16.7

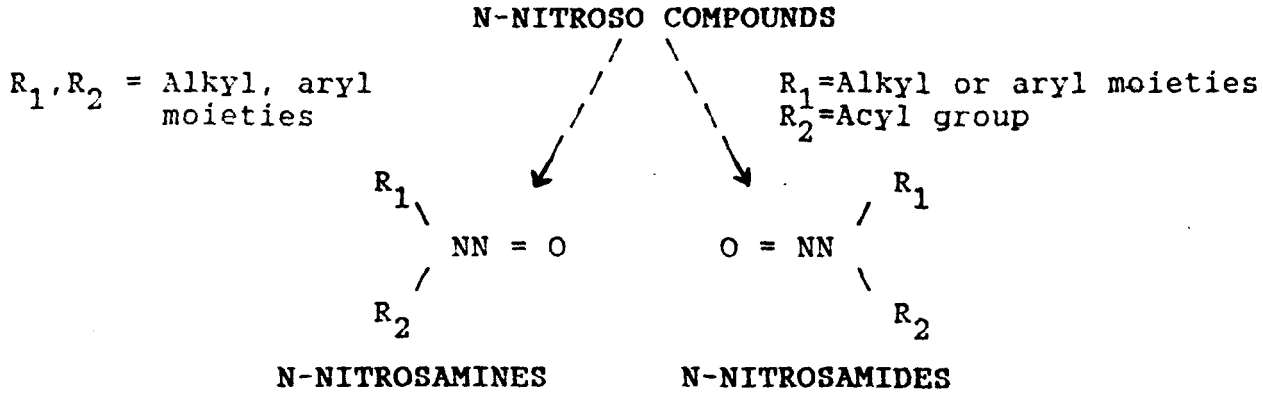
[a] Apart from the amino nitrogen
[b] Including amines with 3 or more *N*-nitroso groups
[c] Direct-acting carcinogens
[d] Indirect-acting carcinogens

Symmetrical dialkylnitrosamines are further sub-classified into *(a)* unsubstituted *(b)* *C*-oxidised *(c)* α-acyloxy, and *(d)* others (miscellaneous or substituted). Asymmetrical dialkylnitrosamines are similarly sub-classified into *(a)* unsubstituted *(b)* *C*-oxidised, and *(c)* others. Cyclic nitrosamines include amino rings of various sizes ranging from four to nine atoms. They are sub-classified into *(a)* unsubstituted *(b)* *C*-oxidised, *(c)* those having another heteroatom in the ring, and *(d)* miscellaneous. Some cyclic nitrosamines possess two or more *N*-nitroso groups and fall under the heading of dinitrosamines. *N*-Nitrosamides and their derivatives may be classified as *(a)* *N*-nitrosoureas *(b)* *N*-nitroso-*N'*-nitroguanidines *(c)* *N*-nitrosourethanes and *(d)* *N*-nitrosamides proper with derivatives.

Table I.1 gives a new classification of *N*-nitroso compounds according to structures, indicating the number tested, the number testing positive for animals carcinogenicity and the number testing negative. *N*-nitrosamines are subdivided into dialkylnitrosamines, cyclic nitrosamines and dinitrosamines, representing the largest group of tested *N*-nitroso compounds. Dialkylnitrosamines are further subclassified as symmetrical or asymmetrical depending upon whether the two alkyl groups R_1 & R_2 are identical or not.

Here the classification method defers somewhat from that of Preussmann & Stewart (1984) who defined symmetry in terms of the number of carbons present in each alkyl group, regardless of actual structure, as will be described later in chapter VI. This new classification strategy serves to demarcate more efficiently

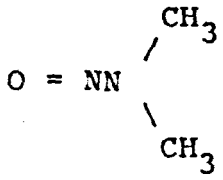
Figure I.4 Chemical structures in the new classification scheme



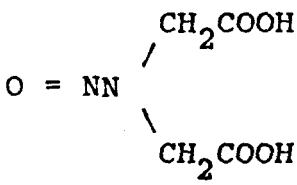
1.Dialkylnitrosamines (DRNA)

(A) Symmetrical DRNA

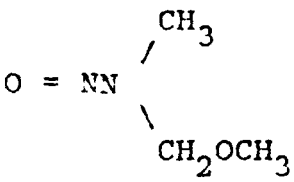
(a) Unsubstituted



(b) C-Oxidised

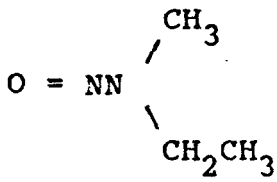


(c) C-substituted

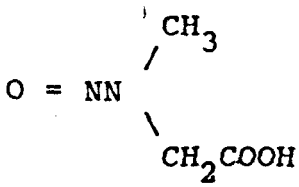


(B) Unsymmetrical DRNA

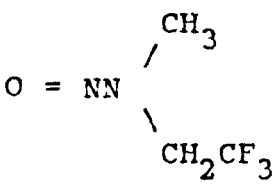
(a) Unsubstituted



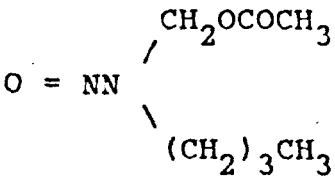
(b) C-Oxidised



(c) C-substituted



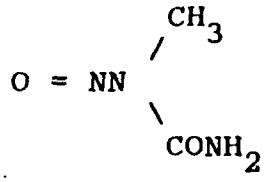
(d) α-acyloxyalkyl



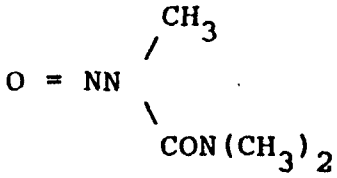
B. N-NITROSAMIDES

1.N-nitrosoalkylureas

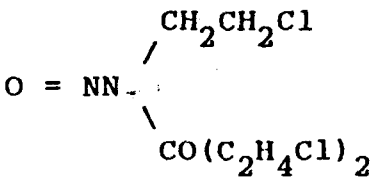
(a) Monoalkylureas



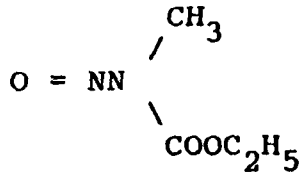
(b) Dialkylureas



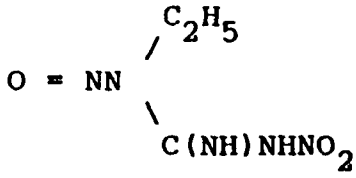
(c) Trialkylureas



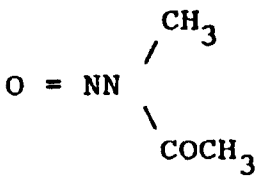
2. N-nitrosourethanes



3. N-nitroso-N'-nitroguanidines

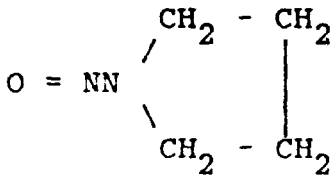


4. N-nitrosamides Proper

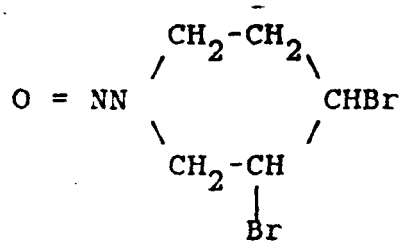


3.CYCLIC NITROSAMINES

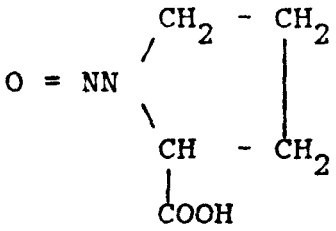
(a) Ring unsubstituted



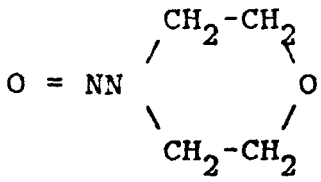
(c) Ring C-substituted



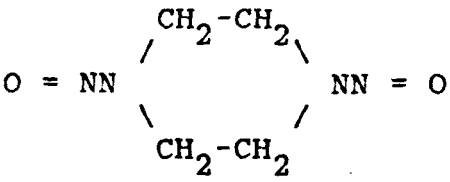
(b) Ring *C*-Oxidised



(d) Ring with hetero-atoms



4. DINITROSAMINES



N,N'-Dinitrosopiperazine

between hepatic and oesophagal carcinogens in rats. The alkyl groups in a dialkylnitrosamines may be unsubstituted or substituted (modified), the most common substitutions being the introduction of oxygen bearing groups. Note that the term "alkyl" is used here in a very broad sense since actually the group may be an alkyl, ararkyl, aryl or heterocyclic groups.

This new classification scheme given above reveals the facts that out of the 332 different NOC tested for carcinogenic activity in various animals, 87% showed positive activity, while 13% were found to give a negative response. This accounts for 176 number of *N*-nitrosamines found active out of the 216 indirect-acting compounds tested and 110 number of *N*-nitrosamides found active out of the 118 number of direct-acting compounds tested in laboratory animals.

Figure I.3 presents the chemical structures of the various classes of *N*-nitroso compounds giving examples of well-studied active members of each class.

I.5 Aims and Objectives of the Research Work

The phenomenon of carcinogenesis by *N*-nitroso compounds is one of the best-studied in cancer research (O'Neill *et al.*, 1984; Bartsch *et al.*, 1981). This dissertation incorporates a body of work done towards investigating some of the experimentally observable features of this phenomenon in terms of the microscopic and mechanistic elements of the process, through the construction of mechanistic models at the molecular level, and using the tools of theoretical chemistry, *viz.* empirical as well as quantum chemical methods like the MNDO and AM_1 methodologies

of the MOPAC package (Stewart, 1983). The main aims and objectives of this research thesis work may be put forward as follows:

1. To evolve a mechanistic model for criteria governing presence or absence of carcinogenic activity in *N*-nitroso compounds.
2. To evolve a mechanistic model for criteria determining in relative carcinogenic potency among *N*-nitroso compounds.
3. To evolve a mechanistic model for criteria involved in choice of target organ for tumourigenesis by *N*-nitroso compounds.

CHAPTER TWO

BIOCHEMICAL MECHANISMS FOR N-NITROSO CARCINOGENESIS

Contents:

II.1 *Understanding Cancer in Molecular Terms*

As mentioned in Chapter One (Sec. I.2) the phenomenon of carcinogenesis can be treated by chemical and theoretical methods only if the process is describable in precise molecular terms. Unless a sufficiently clear molecular basis is put forward for the phenomenon (either through the results of experiment or through proposal of a viable hypothesis), it becomes impossible for the phenomenon to be accessible or amenable to study by the quantum theory of molecular structure interaction, or even by empirical approaches invoking the role of molecular structure and interaction.

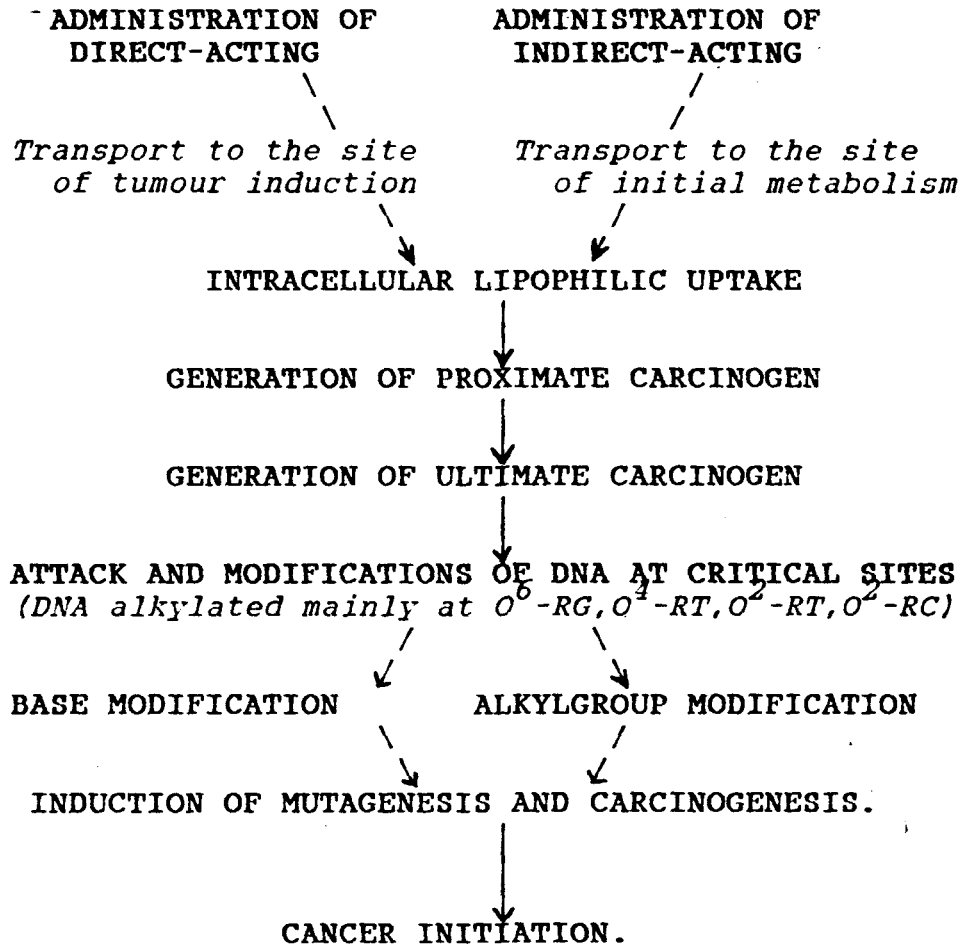
It is a credit to the combined efforts of a large number of researchers (drawn from the rank of organic chemists, biochemists, toxicologists, and molecular biologists) that the phenomenon of chemical carcinogenesis has been much elucidated in molecular terms. This is particularly true for carcinogenesis by *N*-nitroso compounds and alkylating agents. By studying the phenomenon at the molecular level employing appropriate theoretical methods and concepts, the way is paved for understanding carcinogenesis at the commonly observable and macroscopic levels. This implies that the elucidation of the process at the microscopic and molecular levels can often yield valuable insight into the process at higher phenomenological levels.

Referring to the hierarchy levels of understanding of the cancer process (Sec. I.2), the conducting of a study at the most fundamental and irreducible leval (*viz.* the quantum chemical level) can furnish insight at progressively higher levels on the phenomenological scale. For the purpose of this dissertation, study at the quantum chemical level will be shown to be very fruitful in shedding light on some of the salient and observable features of the phenomenon *viz. (a)* the presence/absence of carcinogenic activity *(b)* variations in relative carcinogenic potency, and *(c)* the choice of target organ for tumourigenesis.

II. 2 *Sequence of Mechanistic Events in NOC Carcinogensis*

Much experimental work has been gone into elucidating the precise molecular events which are likely to be involved in

Scheme II.1: Mechanistic sequence of molecular events of chemical carcinogenesis



carcinogenesis by *N*-nitroso compounds. These findings have at large only served to confirm and validate the two key theories currently used to describe chemical carcinogenesis, *viz.* the ultimate carcinogen theory and the somatic mutation theory, which provide the two key pivots around which the modern molecular understanding of chemical carcinogenesis revolves.

The mechanistic link between the ultimate carcinogen and the somatic mutation theory lies in the role played by the ultimate carcinogen to critically modify the proto-oncogene, thereby transforming it to its carcinogenically active forms. The mechanistic basis afforded by these two theories taken together leads to proposal of the sequence of molecular events for *N*-nitroso carcinogenesis as given in Figure II.1, and indicated step-wise as below:

(a) Administration of parent carcinogen to the host system: Various routes for administration of the parent compound have been utilised for the different animal species tested, primarily rats and mice. Common administration routes include oral intake (po), inhalation, intravenous injection (iv), intraperitoneal injection (ip), subcutaneous injection (sc), and topical application. A variety of dosage schedules may be pursued, which would have their influence on the carcinogenic effects produced, notably, the time period required for tumours to develop and possibly the tumour yield, too. In many cases, the administration routes are of importance in determining the site of tumour induction, mainly because of the way in which they serve to transport the parent *N*-nitroso compounds to the appropriate site.

(b) In vivo *transport of the carcinogen to the site of metabolism and tumour induction:*

The path followed during the transport, and the areas reached within the animal, depend upon the mode of administration. For direct-acting NOC, the organs affected may be near or distant from the site of administration, depending upon the *in vivo* stability of the compound. Oral intake (po) of *N*-nitrosamides leads to decomposition along various segments of the alimentary canal in rodents, where the *p*H of the fluid along the segments would be a factor affecting stability of the parent *N*-nitroso compound.

For indirect-acting *N*-nitroso compounds, it may be expected that the sites affected should possess the necessary metabolising enzyme systems in their tissues. It may be noted that not only the parent compounds, but their metabolites (stable / metastable) may also be transported away from the site of initial metabolism to other organs.

(c) Intracellular lipophilic uptake to concerned organelle:

Since permeation of the lipid bilayer of the cell-wall is necessary for *N*-nitroso compounds to reach the organelle more intimately involved with the intracellular fate of the carcinogen (*viz.* the mitochondria and eventually the cell nucleus), lipophilicity of the parent NOC emerges as a factor influencing the xenobiotic effects of the compound. This has formed the basis for the QSAR Hansch approach (Hansch, 1976) to correlating lipophilicity of NOC with their carcinogenic activity (Singer *et al.*, 1977).

(d) Generation of proximate carcinogen(s):

For the direct-acting *N*-nitrosamides, the proximate carcinogen is thought to be the alkanediazohydroxide, the product of catalysed hydrolysis. For the indirect-acting *N*-nitrosamines, the postulated proximate carcinogens include the α-hydroxyalkylnitrosamine, the monoalkylnitrosamine, and the alkanediazohydroxide. All of these are further subject to spontaneous conversion to the ultimate carcinogen. Because of their relative instablity (although not as unstable or as reactive as the ultimate carcinogen species), they should be formed fairly close to the nucleus and to the DNA, so that they are not exhausted en route as they approach the critical targets on DNA.

(e) Generation of ultimate carcinogen(s):

The proximate carcinogen decomposes or undergo facile dehydration to yield the highly reactive and unstable ultimate carcinogen species. The opinion adhered to here is that the alkanediazonium ion furnishes the best choice among various alternatives for the actual ultimate carcinogen species. The attack and modification of cellular DNA is actually brought about by this highly reactive ultimate carcinogen species.

(f) Attack and modification of DNA at the critical sites:

The generation of the ultimate carcinogen species is then followed by the attack and modification of DNA at the critical sites. The alkanediazonium ion goes on to attack and modify DNA at the critcal sites which are nucleophilic in nature. Depending upon the groups present in the ultimate carcinogen, the modification may take the form of alkylation, aralkylation or

arylation. The critical site on the proto-oncogene are most likely to be oxygen sites, like O6-guanine or O4-thymine.

(g) Adoption of mutagenic property by modified DNA:

The mechanism of key mutation involved in *N*-nitroso carcinogenesis is most likely to be of the point mutational type, *viz.* base substitutions. The actual structural features responsible for the adoption of mutagenic property by DNA following modification is here to be the abstraction of the Watson-Crick protons of modified guanines and thymines residues.

(h) Activation of the proto-oncogenes:

The deprotonated DNA base has a potential to undergo aberrant or unusual base pairing, resulting in incorporation of the wrong base during DNA replication. This engenders base-substitutions, which type of mutation has been discovered in many cases to be responsible for the activation of proto-oncogenes to their carcinogenically active form (Varmus, 1984).

(i) Expression of activated oncogene(s):

The critically activated oncogene(s) are then expressed during transcription and protein synthesis as the oncoprotein products, which go on to participate in the macromolecular changes involved in the cellular and molecular biology of the cancer cell. In general more than one oncogene is involved.

This scheme represents in sketches the general sequence of molecular events, begining from the route of administration till the stages of DNA repair system for both the direct-acting and the indirect-acting *N*-nitroso compounds. The only difference

between the two types of carcinogens is that the chemically unstable direct-acting *N*-nitrosamides undergo an acid-base hydrolysis or spontaneous decomposition (Dutton & Heath, 1956; Brouwers & Emmelot, 1960; Magee & Barnes, 1956; 1967) to yield the proximate and ultimate carcinogen species, while the chemically stable indirect-acting carcinogen is activated by enzyme-mediated metabolism (Malling, 1971; Gletten *et al.*, 1975) to yield the proximate and ultimate carcinogen species.

In the preceeding sequence of events, the conferred mutagenicity or carcinogenicity may be due to point mutation by direct alkylation of nuclear DNA (Boyland *et al.*, 1968; Dontenwill, 1968), or due to incorporation of alkylated nucleotides in DNA (Saffhill & Fox, 1980; Chetsanga & Lindahl 1979; Laval *et al.*, 1981; Yarosh, 1985) or due to change in deoxynucleotides 5-triphophate pools (Pegg *et al.*, 1983; Ahmed & Laval, 1984; Dolan *et al.*, 1984; Singer, 1986).

II. 3 Administration and Transport of Carcinogen in vivo

The varieties of administration routes employed for intake of the *N*-nitroso compound by the host animals is one factor involved in determining the target organ sites for tumour induction. These effects are discussed in detail in Sec. VI.2. The dosage schedule employed is of possible consequence for the degree of carcinogenic potency manifested. Druckrey *et al.* (1963) have shown a linear dependency of the carcinogenic total dose (D_{50}) of diethylnitrosamine on the daily dosage plotted on log-log coordinates, the carcinogen being administerd to *BD II* rats. The same study also demonstrated a linear dependency of the median

induction time (d_{50}) on the daily dosages of diethylnitrosamine, plotted on log-log coordinates. Differences have been noted in carcinogenic effects depending on whether a single dose, low chronic dose or multi-generation regimes are employed. Dose-response studies on *N*-nitroso compounds have been critically discussed by Schmahl (1980). The most extensive quantitative dose effects studies carried out by Druckrey *et al.* (1963) produce results which fit the formula of Druckrey (1943) and of Druckrey & Kuppfmuller (1948) given below:

$$dt^n = \text{constant}$$

where *d* is a total dose, *t* the median induction time, the factor *n* for diethylnitrosamines being about 2.6.

The distribution route followed by the *N*-nitroso compounds depends to a large extent upon the mode of administration, which effects are discussed in greater detail in Sec. VI.2. *In vivo* transport of the *N*-nitroso compounds following administration may here be described as being of two kinds, *viz. pre-metabolic* and *post-metabolic*. The former refers to transport of carcinogens to the initial site of metabolism (which however may in some cases be also the site of tumour induction). An example is the transport of *N*-nitrosamines from the alimentary canal to the liver by the hepatic portal vein, following oral intake.

When the site of initial metabolism does not correspond to the site of tumour induction, post-metabolic transport can occur. This entails initial metabolic transformations to a fairly stable metabolite (proximate carcinogen) which may then be retransported

to another organ or site, where tumour induction may then occur. Post-metabolic transport may be examplified the case of bladder carcinogenesis by dialkylnitrosamines containing *n*-butyl groups (Blattmann & Preussmann, 1974). Here the initial metabolism occurs in the liver where *w*-butyl hydroxy derivatives are produced and retransported by the process of excretion to the bladder. In the bladder further metabolism of the *w*-hydroxy butyl derivatives takes place (presumably by *α*-hydroxylation) to yield the highly reactive ultimate carcinogen).

For direct-acting *N*-nitroso compounds the length of time spent in transport by the carcinogen depends upon its *in vivo* stability. In general at biological *p*H the following stability order may be said to hold: Alkylnitrosourea > *α*-acyloxyalkyl-nitrosamine > *α*-hydroxyalkylnitrosamine. For stabler varieties of direct-acting *N*-nitroso compounds (*viz.* alkylnitroso-ureas), tumour induction may be capable of occuring at organs fairly distant from the site of administration which is one factor certainly responsible for the induction of distal brain and nervous system tumours by alkylnitrosoureas even when administered subcutaneously (IARC Monograph on *N*-Nitroso Compounds, 1979). Less stable varieties of direct-acting *N*-nitroso compounds would in principle be capable of exerting their primary effects only locally around the administration site which is the case for oral administration in rats of *α*-acyloxy alkylnitrosamines (resulting in tumours of the forestomach) (Weissler *et al.*, 1978) and also for subcutaneous administration in rats of alkylnitroso-*N*-nitroguanidines (resulting only in local tumours of the subcutis) (Sano *et al.* 1978).

II.4. Metabolism of Indirect-Acting N-Nitroso Compounds

Indirect-acting *N*-nitroso compounds (including most dialkylnitrosamines and cyclic nitrosamines) are susceptible to metabolic transformation through hydroxylation or oxidation of carbon sites by cytochrome P_{450} or mixed function oxidase systems. Metabolism may be carcinogenically activating or deactivation of which only the former is dealt with in this section . For the latter see section II.8.

For dialkylnitrosamines, the following activation metabolic routes may be noted.

(a) The α-hydroxylation pathway:

Much evidence exist for proposing enzymatic α-hydroxylation as furnishing a major activating metablic pathway for dialkylnitrosamines. This basically involves hydroxylation of an α-carbon hydrogen bound by the oxidase systems to yield an unstable α-hydroxy alkyl derivatives. Figures II.2 & II.3 depict this activation pathway of dialkylnitrosamines and cyclic nitrosamines where the initial step involves abstraction of a hydrogen atom from the α-carbon by the triplet oxygen of the P_{450} intermediate to give a radical intermediate. This undergoes recombination with the OH$^{\cdot}$ radical to yield the α-hydroxyalkyl metabolite. This is capable of undergoing spontaneous decomposition through concerted reaction involving six membered transition state to yield the alkanediazohydroxide (or tautomeric monoalkylnitrosamine) and the corresponding aldehyde.

Current theoretical and mechanistic considerations (Reynolds & Thomson 1986; Loew *et al.*, 1983; Druckrey *et al.*, 1968), favour

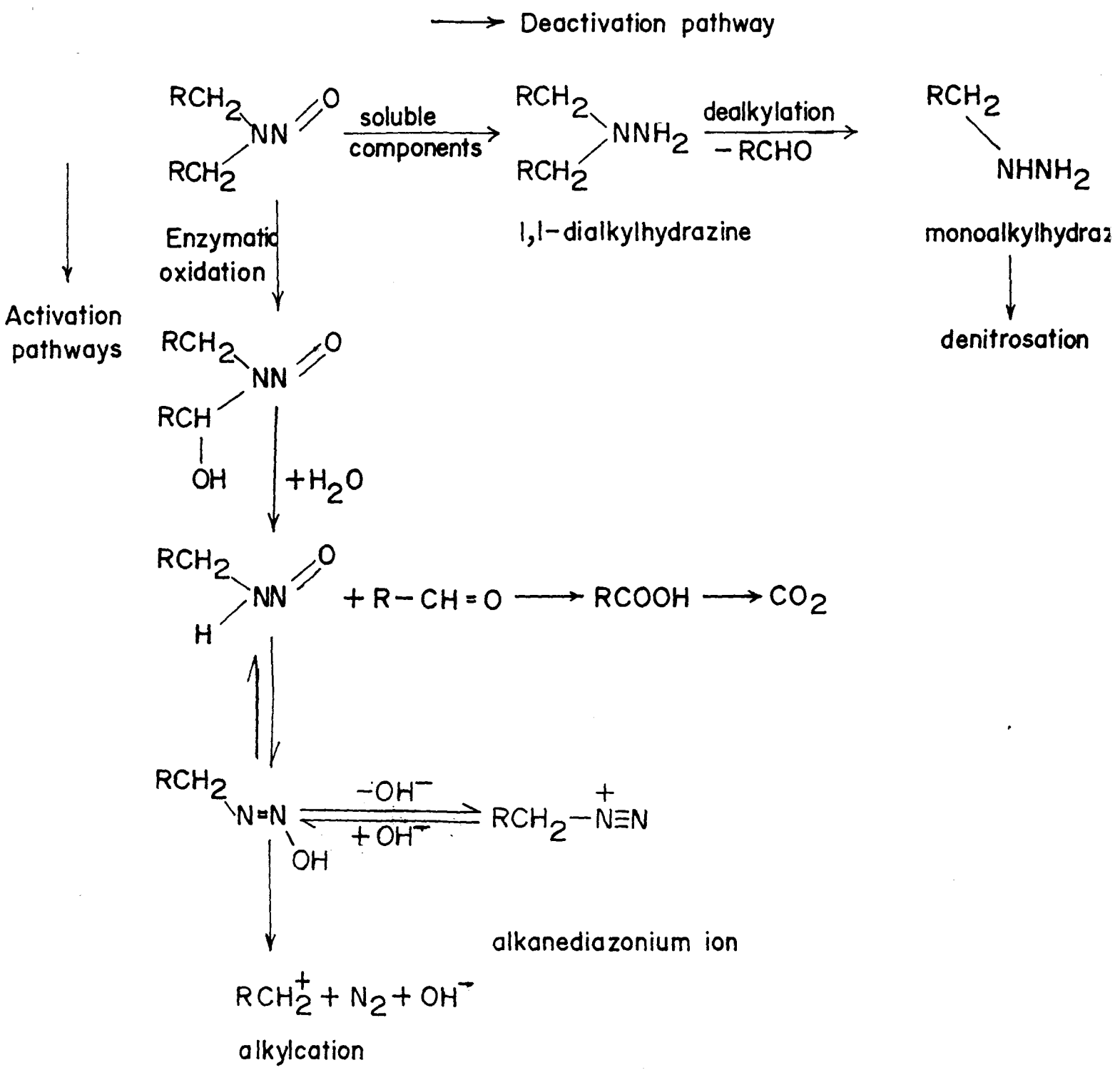
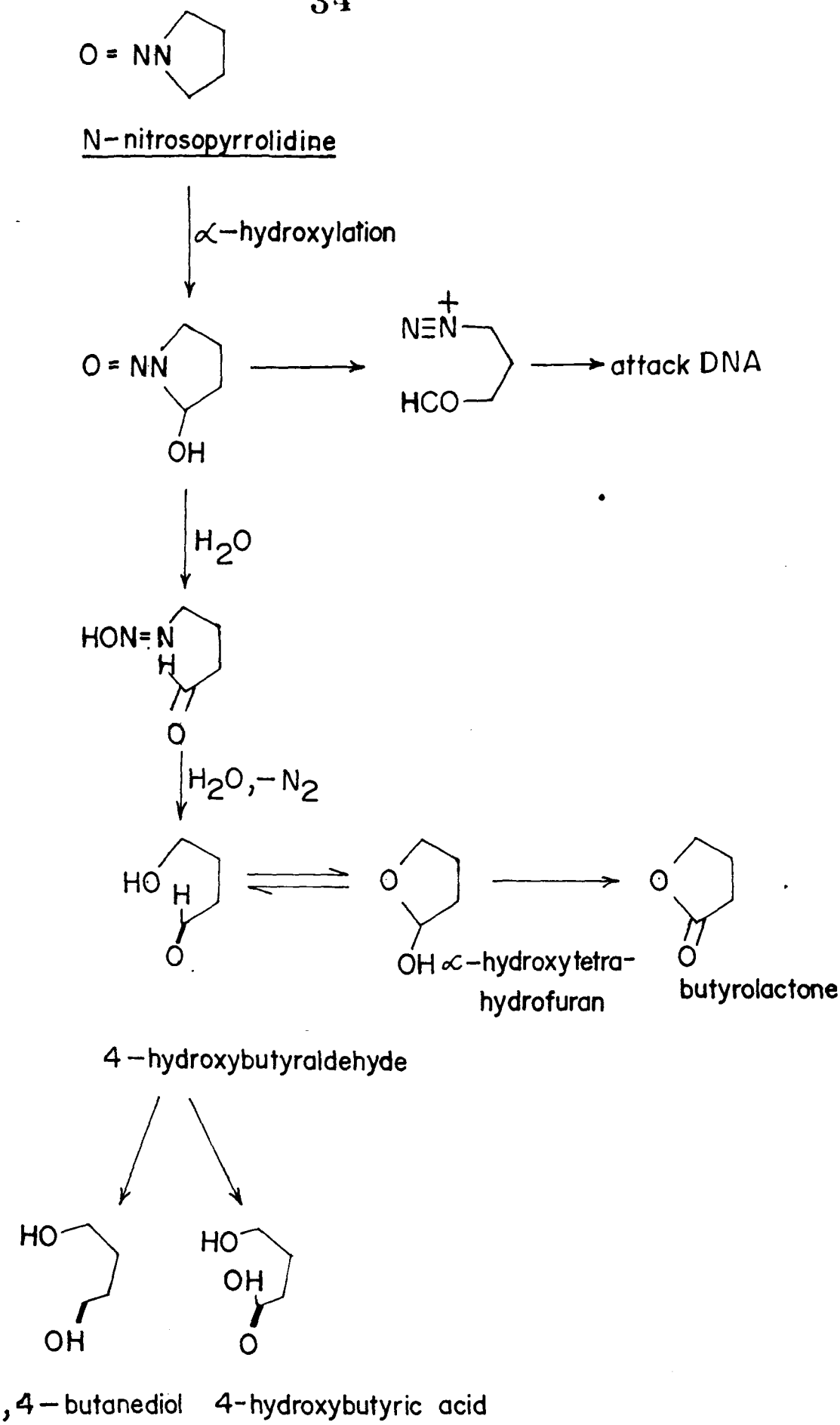


Fig II.2: Enzymatic Bioactivation pathway and Detoxification pathway of Dialkylnitrosamines.



FigII·3 : Enzymatic Bioactivation pathway of cyclic nitrosamines

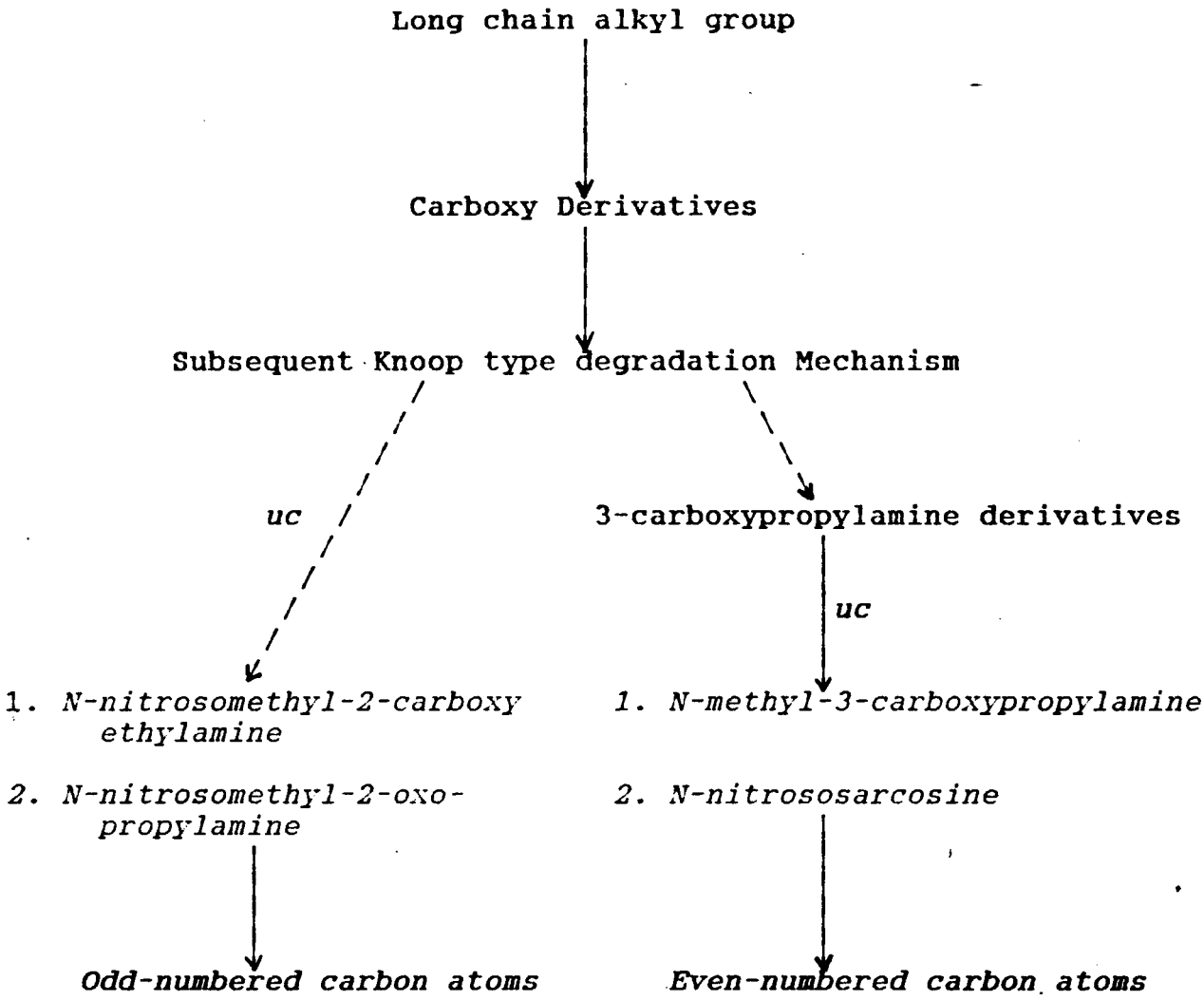
involvement of the alkanediazohydroxide rather than the monoalkylnitrosamine Lyngdoh (1994) has shown using molecular orbital calculations that the α-hydroxyalkyl metabolite and alkanediazohydroxide both possess a low level of α-carbon electrophilicity comparable to the inert parent nitrosamines. This would rule out the identification of these species as ultimate carcinogen candidates, being more properly classifiable as proximate carcinogens. They can undergo futher decompostion via loss of water to yield the alkanediazonium ion. The opinion adhered to here (Lyngdoh, 1994) is that the diazonium ion provides the best choice for the actual ultimate carcinogen or prime DNA-modifying agent in *N*-nitroso carcinogenesis.

Note that, unlike for the β-to ω-hydroxylation pathways, there is no urinary metablite evidence for the α-hydroxylation apart from the detection of aldehydes. This is due to the extreme instability of the α-hydroxyalkylnitrosamine. The expected aldehydes have been identified as urinary metabolites following metabolism of dimethylnitrosamine, di-*n*-propylnitrosamine, di-*n*-butylnitrosamine, methylethylnitrosamine and other nitrosamines (Lake *et al.*, 1976; Park & Archer, 1978; Blattmann & Preussmann, 1977; Farelly, 1980; Schweinsberg, 1979; Chau *et al.*, 1978). The α-hydroxylation activating pathway has been reviewed by Montesano and Magee (1974).

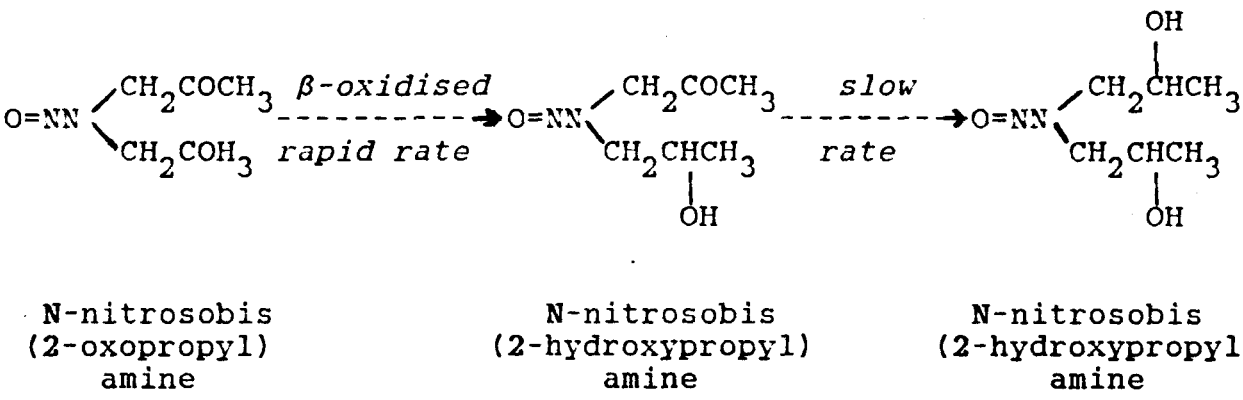
(b) Knoop-type degradation of long chain alkyl groups:

N-nitroso methyl-*n*-alkylamines with long alkyl chains are susceptible to Knoop-type degradation of the long chain in a manner analogous to that of fatty acids by successive removal of

Scheme.II.4: Knoop type degradation of long chain alkyl groups relevant for urinary bladder carcinogenesis.



Scheme.II.5: β-oxidation relevant to pancreatic cancer in Syrian hamsters.



N-nitrosobis (2-oxopropyl) amine

N-nitrosobis (2-hydroxypropyl) amine

N-nitrosobis (2-hydroxypropyl) amine

two carbon fragments following initial *w*-oxidation to the carboxy derivatives. Singer *et al.* (1981) studied this metabolism pathways for members chain length from C_4-(*n*-butyl) to C_{14} (tetradecyl in rats).

The end product of the metabolism (urinary metabolite) from members with odd-numbered chains has a *β*-oxidised *n*-propyl group. Members with even-numbered chain formed *w*-oxidised *n*-butyl derivatives. The post-metablic transport of these metabolites could be responsible for bladder carcinogenesis in rats for even-numbered chains (Suzuki *et al.*, 1981). The Knoop-type degradation pathway of metabolism is given in Figure II.4.

(c) β-oxidation of di-n-propyl nitrosamines:

β-Oxidation of both the alkyl chains are of special relevance for pancreatic tumours in hamsters (Gingell *et al.*, 1976). An example of such case is shown by the metabolism of N-nitrosobis(2-oxo-propyl)amine in hamster as given Figure II.5 below. Structures II & III are found in pancreas, but not structure I (Gingell & Pour, 1978); and II is the major proximate metabolites in pancreatic tumours.

(d) Metabolism of dibutylnitrosamines and related compounds:

The major metabolic pathways of *w*-oxidation leads to selective urinary bladder carcinogen, *viz.*, *w*-hydroxy-*n*-butyl nitrosamines and 3-carboxypropyl-*n*-butylnitrosamines (Okada & Ishidate, 1977; Blattmann & Preussmann 1973; 1974). Once again post-metabolic transport of this *w*-oxidised derivatives via excretion to the urinary bladder is a factor for bladder

carcinogenesis in rats. The metablism of alkyl-4w-hydroxybutyl nitrosamines (R = methyl, ethyl, propyl, *tert*-butyl, pentyl) demonstrated that the presence of *w*-hydroxy butyl or *r*-carboxy propyl moiety is necessary for bladder carcinogenesis (Suzuki *et al.*, 1981). The *w*-oxidised derivatives, after arriving at the bladder, could undergo further *α*-hydroxylation as seen in *(a)* above.

II.5. Decomposition of Direct-acting N-nitroso compounds.

Direct-acting *N*-nitroso compounds include the sub-classes of *N*-nitrosoureas, *N*-nitroso-*N*-nitroguanidines, *N*-nitrosourethanes, *N*-nitrosamides proper and *α*-oxidised alkylnitrosamines. Unlike the indirect-acting nitrosamines, all these compounds (or their immediate hydrolysis products) are characterised by the property of facile conversion via six-membered transtion state to the alkanediazohydroxide (or diazotate) and a species containing an unsaturated carbon. They are all prone to hydrolytic cleavage of the *C-N* bond, resulting in production of the RNNOH molecule. They undergo spontaneous decomposition to yield their reactive ultimate carcinogen species. The decomposition is acid-base catalysed and is presumed to occur through a concerted pathway involving a cyclic transition state. Alkylnitrosoureas and other *N*-nitrosamides are similar in behaviour to *α*-hydroxy-, *α*-acyloxy- and *α*-hydroperoxy derivatives of dialkylnitrosamines (Baldwin *et al.*, 1976; Saavedra, 1979; Mochizuki *et al.*, 1980a, 1980b; Frank *et al.*, 1980). The mechanism of spontaneous decomposition pathway of direct-acting *N*-nitrosamides is portrayed in Figure II.6.

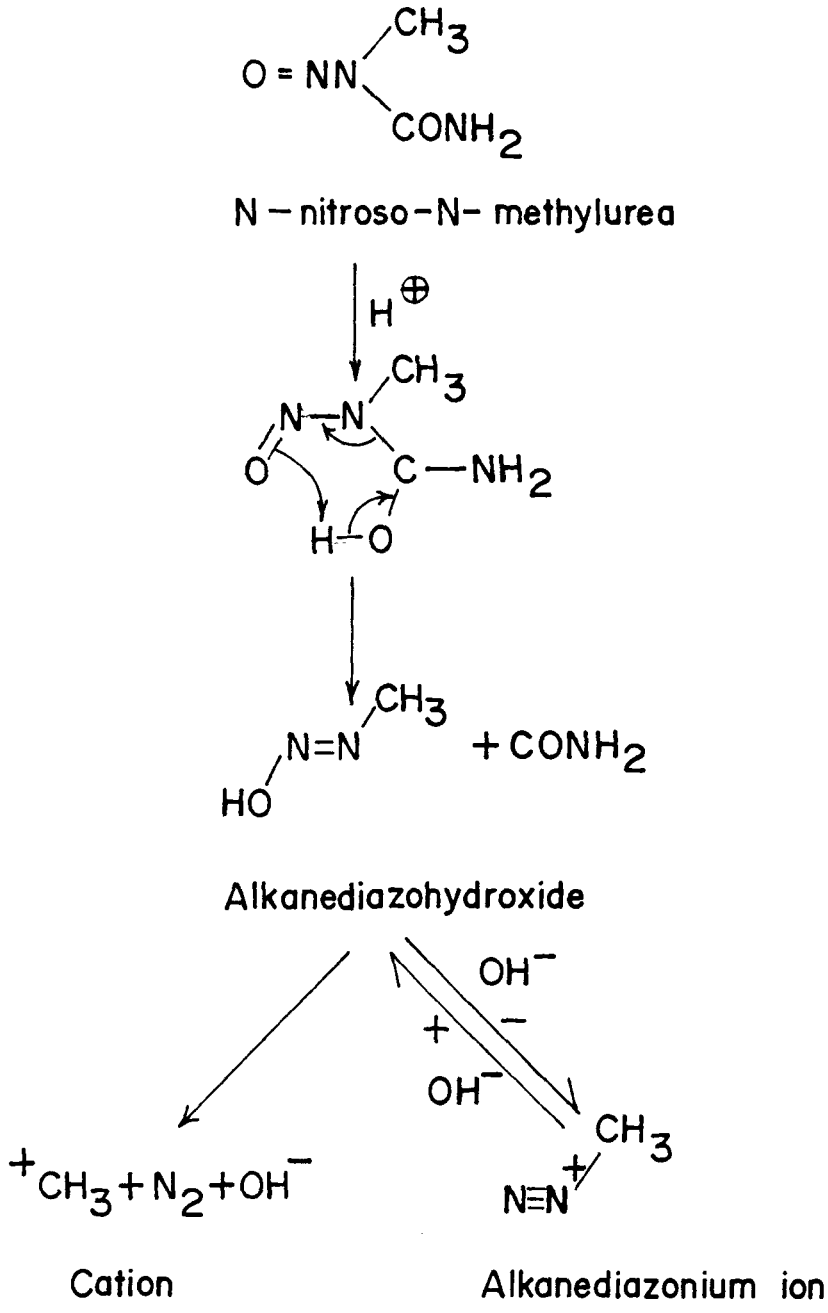
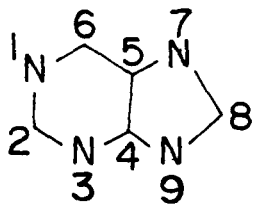


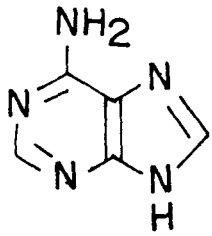
Fig II.6: Acid-base or thiol Catalysed hydrolysis of direct-acting N-nitroso compound.

Parent structure and numbering system



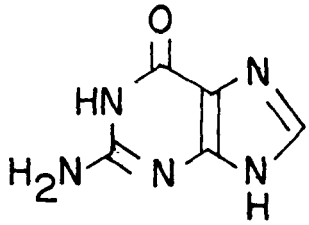
Purines

DNA base

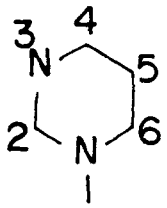


Adenine (A)

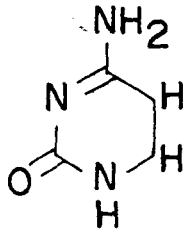
DNA base



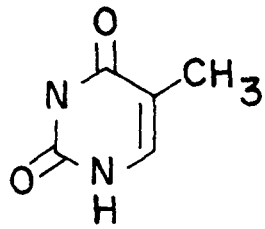
Guanine (G)



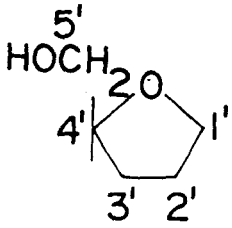
Pyrimidine

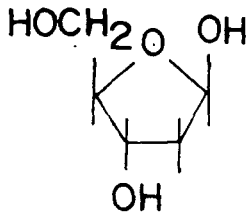


Cytosine (C)



Thymine (T)





B – 2'– deoxyribose
(in DNA)

Fig II.7: Parent structure and numbering system of DNA bases, with abbreviations.

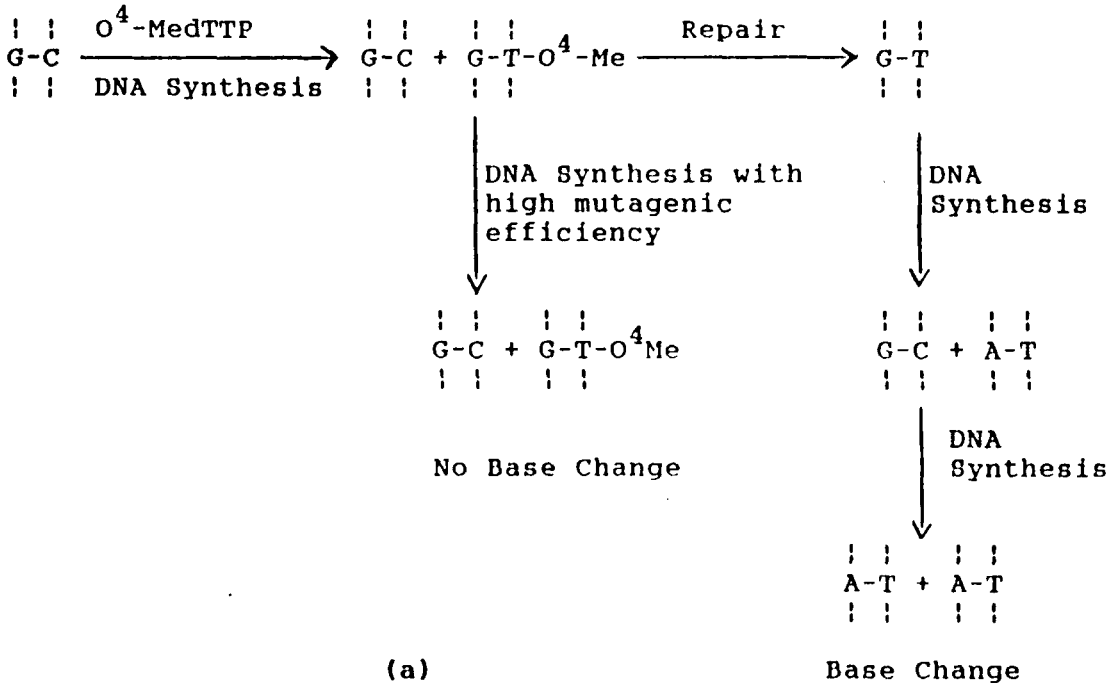
II.6. Attack and Modification of DNA

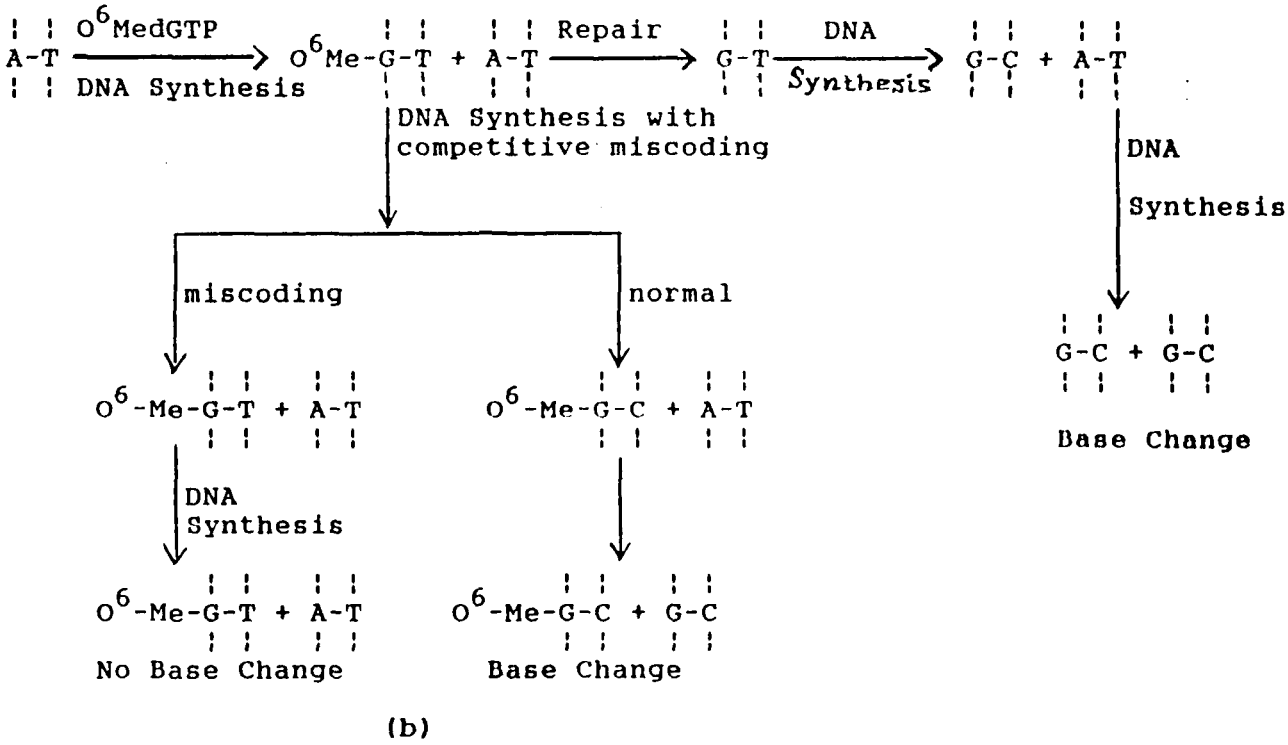
The somatic mutation theory in the context of chemical carcinogenesis would suggest that chemical modification of DNA by the chemical carcinogen via its ultimate carcinogen form is a crucial step along the carcinogesis pathway. For *N*-nitroso compounds, the alkanediazonium ion, as a reactive electrophile, serves to modify DNA at various nucleophilic sites. Some 16 different nucleophilic sites on DNA have been identified as being modified by *N*-nitroso compounds and alkylating agents under *in vitro* and *in vivo* conditions. Direct-acting *N*-nitroso compounds as well as indirect-acting compounds have been studied not revealing marked differences between nitrosamines and nitrosamides. Fig II.7 gives the basic structure and standard atom numbering system for DNA components, *viz.* the DNA bases and sugar phosphates moieties.

Tabulations of DNA sites modified by *N*-nitroso compounds and alkylating agents have come out in a number of reviews (Singer, 1975 & 1976; Singer *et al.*, 1978; Richardson *et al.*, 1987; Pegg & Nicoll, 1975). The sites which have been isolated as alkylation targets on DNA *in vivo* and *in vitro* include the phosphate group oxygens, the O^2 atom of deoxyribose, the N^1, N^3, N^7 sites of adenine, the N^3, O^6, and N^7 sites of guanines, the O^2- and O^4-sites of thymine and O^2 and N^3 sites cytosines.

The alkanediazonium ion formerly employs an S^N2-alkylation mechanism for modification of nucleophilic sites on DNA. Depending upon the identity of the alkyl groups present, a greater or lesser degree of S^N1-character would also be present in the alkylation mechanisms. Ford and Scribner (1990) have shown

Table II.8 Miscoding pathways arising from the incorporation of (a) O^4-MedTTP and (b) O^6-MedGTP during DNA synthesis.





that the degree of S^N1-character in the transtion state is related to the degree of *O*-selectivity of DNA modification. Methylating *N*-nitroso compounds with lower S^N1-character of the methanediazonium ion leads to a high proportion of *N*-alkylated products, notably N^7-methylguanine. For ethylating *N*-nitroso compounds the proportion of *O*-alkylated products is higher. The ratio of *O*-alkylation to *N*-alkylation is highest for *sec*-alkylating agents.

The initial product of DNA modification by the diazonium ion is a cationic alkylated base. This may or may not loss a Watson and Crick protons to yield a potentially mutagenic species. N^7-alkylguanines retain their Watson-Crick protons at biological *p*H and are thereby mutagenic. O^6- alkylguanines and O^4 alkylthymines readily loss their Watson-Crick protons at biological *p*H to yield a potential mutagenic species which incorporates the wrong base during DNA replication.

An example of the miscoding or misincorporation of DNA bases during DNA synthesis arising from the incorporation of O^4-MedTTP and O^6-MedGTP is portrayed in Figure II.8. The promutagenic and procarcinogenic significance of *O*-alkylated residues as opposed to *N*-alkylated residues leads to the inference that *O*-selectivty of DNA modification would be an important factor for carcinogenesis and mutagenesis. Note that the direct alkylation of DNA itself may not be the only causative factor for mutagenesis and carcinogensis, since these could conceivably be brought about by incorporation of alkylated nucleotides in the DNA during synthesis or by modification of deoxynucleosides 5'-triphosphate pools (Pegg *et al.*, 1983; Singer, 1986).

II.7 Role of Oncogene(s) for N-nitroso Carcinogesis

Of the three mechanisms reviewed by varmus (1984) for the activation of proto-oncogenes the point mutation mechanism is the one that best fit into the carcinogenesis pathways for *N*-nitroso compounds. Proto-oncogenes activations has been well documented for the *ras*-family of oncogenes in human and animal cancers (Ames, 1979; Varmus, 1984), some 18 cases having been discovered by 1984. More recently, the *neu* oncogenes in rat neuroblastoma has also been found to be activated by point mutational mechanism. The critical mutation in the transforming *ras*-gene involved just single base-substitution at the 12th, 59th and 61st codons only so that the amino-acid sequence of the oncoprotein is changed only at one point. Kim *et al* (1961-62) have demonstrated how this small changes have very significance effects on the biological activity of the *ras*- oncoprotein. The *ras*-oncogene family codes for proteins having GTPase activity (Teich, 1986).

The base-substitution characterised for this activation of the *ras*-oncogene family include *G-C* to *A-T* and *A-T* to *G-C* transition as well as transversion *G-->T*, *G-->C*, *C-->A* and *A-->T*. This fit in well with the well documentated *N*-nitroso compounds ability to induce point mutation of a base-substituted character. This ability of *N*-nitroso compounds and alkylating agents to induce point mutation owes its origin to the pro-mutagenic properties of *O*-alkylated base residues O^6-alkyl guanines and O^4-alkyl thymines as demonstrated by *in vivo* and *in vitro* studies on the bearing properties of nucleic acid template incorporating this alkylated residues systems (Loveless, 1969; Gerchman & Ludlum, 1978; Mehta

& Ludlum, 1978; Bhanot & Ray, 1986; Singer *et al.*, 1978; Abbott & Saffhill, 1977; Singer *et al.*, 1986; Preston *et al.*, 1986).

Brodeur *et al.* (1984) have demonstrated the ability of *N*-nitroso methylurea to activate the *N-ras* proto-oncogene in mice tumour by a single point mutation. Direct activiation of *C-ha-ras1* proto-oncogene by *in vitro* reaction with methyl, acetoxy methylnitrosamines has been demonstrated by Hirani-Hojatti *et al.* (1986). The above array of evidence points strongly to the inference that *N*-nitroso compounds can act as carcinogen through point mutational activation of proto-oncogenes. While this may not ruled out the possibility of other mechanism for *N*-nitroso carcinogenesis, the weight of current evidence favours the point mutation model for NOC carcinogensis. This is the model that adhered to as a basis for the work embodied in this dissertation.

II.8 Some Deactivating Pathways:

In conjunction with the activating pathway described above, a number of potential deactivating pathways also operate. This introduces the element of competitions, the extent of which would determine the degree of manifestation of the carcinogenic effect. The following deactivating pathways may be noted:

(a) Metabolic alteration of N-nitrosamines:

This can be the outcome of ezymatic reduction of the *N*-nitrosamines bond to form the correspondent hydrazine which appear to be inactive as carcinogen. Such reaction would involved a cytochrome P_{450} enzymes (Crosby & Sawyer, 1976; Lyle *et al.*, 1979) demonstrated for dimethylnitrosamines, *N*-nitrosomorpholine. Metabolic denitrosation of nitrosamines (Appel *et al.*, 1979;

1980) would produce the parent amine and nitrite, which is deactivating, since the parent amine is non-carcinogenic. *In vivo* denitrosation of *N*-nitroso-*n*-methyl-*N'*-nitroguanidines (Kawachi *et al.*, 1970) led to recovery of a significant fraction of the injected dose as inactive methylnitrosoguanidines.

(b) β-to ω-hydroxylation of dialkylnitrosamines:

Unlike the product of α-hydroxylation of dialkylnitrosamines the β-to ω-hydroxylated derivatives are generally stable metabolites which can be detected in the urine of test animals. Such metabolites and their oxidised derivatives have been obtained for diethylnitrosamine, dipropylnitrosamine, diisopropylnitrosamine, and dibutylnitrosamine. Their stability has compared to the α-hydroxy derivatives is due to their inability to form a facile six-membered transition state for degradative decomposition. In general, they would be excreted out of the living system in the form of soluble ester like glucoronides. Since they do not yield directly any ultimate carcinogen, the formation of these products lead to a loss of the potential alkylating species, and thus may be described as deactivating. Exception may be provided by the cases of ω-oxidised *n*-butyl derivatives and β-oxidised *n*-butyl derivatives and β-oxidized *n*-propyl derivatives as noted above in sect II.4.

(c) Hydrolytic loss of ultimate carcinogen:

Owing to the extreme reactiviy of the ultimate carcinogen, it may suffer a lesser or greater degree of depletion through hydrolysis by the aqueous cellular environment before it reaches

the critical target in DNA. This deactivating route of hydrolysis to inactive alcohols is thereby a factor leading to loss of carcinogenic potency, and is dealt with in chapter V.

(d) Cyclisation of the UC to inactive products:

In many cases the potential ultimate carcinogen would be capable of undergoing intramolecular cyclisation which could lead to formation of non-carcinogenic products. If such reactions are facile enough, the result could even be complete loss of carcinogenicity. These cases are dealt with further in Chapter III.

(e) Attack of non-mutagenic sites in DNA:

While certain oxygen sites like O^6-G and O^4-T are of promutagenic and carcinogenic relevance when alkylated (Singer *et al.*, 1978; Margison & O'Connar, 1979), there exist no evidence for the mutagenic or carcinogenic role of *N*-alkylated products like N^3- and N^7-alkylguanines and N^3 and N^7 alkyladenine (Ludlum, 1970; Laval 1977; Ishiwata *et al.*, 1979). This may be interpreted to mean that sufficiently high proportions of *N*-alkylations verses *O*-alkylations would be a factor detracting from the carcinogenic effect. The S^N2-character of the mechanism play the role here, as will be dealt with further in chapter IV.

(f) Deactivating role of DNA repair:

Alkylated DNA components are subject to repair by various mechanisms. In general *N*-alkylated DNA nucleosides are repaired by the action of the glycosylases enzymes that remove the entire base or nucleosides leaving an apurinic or apyrimidinic sites (Laval, 1977; Chetsanga *et al.*, 1979; Laval *et al.*, 1981). O^6-

alkyl guanines however may repair by an alkyltransferase enzymes which directly transfer only the alkyl groups to a cysteine residues in the enzyme (Schendel & Robins, 1978; Yarosh, 1985). There is evidence that repair of O^4-alkylthymines is much slower than O^6-alkylguanine in bacterial and mammalian cells, leading to the inference that the alkylation at O^4-thymine site may be more significant for mutagenesis and carcinogenesis than alkyaltion at O^6-guanine (Singer, 1986; Singer *et al.*, 1981). Owing to the pro-carcinogenic and pro-mutagenic significance of this *O*-alkylated bases, it would be alkyltransferase repair capacity rather than glycosylase repair capacity which has more direct bearing upon carcinogensis.

Error-free repair of this DNA lesions would thereby be manifested as a deactivating pathway, while an error-prone mechansim may prove to be an activating pathway for the induction of tumours. Differences in repair capacity between various organs would also obviously be a factor determining the choice of target organ for carcinogenesis,and is dealt with further in chapter VI.

CHAPTER THREE

MECHANISTIC CRITERIA FOR PRESENCE OR ABSENCE OF CARCINOGENIC ACTIVITY IN *N*-NITROSO COMPOUNDS. ROLE OF THE PARENT, PROXIMATE AND ULTIMATE CARCINOGEN SPECIES

Contents:

III.1 Screening Tests for Carcinogenic Activity

N-Nitroso compounds (NOC) display a wide variety of responses in the various animal systems in which they have been tested for carcinogenicity. In the literature till 1984 , as many as 334 different NOC had been tested in various species and strains of animals for carcinogenicity employing various modes of administrations routes and dosage schedules. In the comprehensive survey published in 1984, Preussman and Stewart (1984) compiled the results of long-term screening tests using animals performed on some 332 different (NOC) to examine for presence or absence of carcinogenic activity. The majority of compounds tested (87 %) showed a positive carcinogenic response, while 13 % were negative (or presumed nearly so).

This accumulation of evidence points to the high carcinogenic

risk associated with NOC as a general class. However, the observation that certain NOC could be industrially useful (eg. as solvents and precursors - Mangino *et al.* (eds.), 1982; Spiegelhalder *et al.*, 1982; Drews *et al.*, 1957) has initiated the search for NOC which are not carcinogenic in animals, and hopefully not in man too (Chu & Magee, 1981; Garcia *et al.*, 1972; Lijinsky & Reubers, 1982; Lijinsky & Taylor, 1977).

Long-term screening for carcinogenicity using laboratory animal is very expensive, laborious and time-consuming, although it is the only definitive test so far available for carcinogenicity. The large cost and effort involved in such tests has prompted efforts to devise cheaper, short-term tests which could provide reliable alternatives. Short-term screening methods commonly used include the *in vitro* cell culture transformation tests (Heidelberger, 1977; Kakunaga, 1977; Cooper, 1982; Newbold & Overell, 1983; Harris, 1979; Shay *et al*,. 1981) and the mutagenicity assays like the Ames *Salmonella* mutant reversion test (Ames *et al.*, 1973; 1975; 1979). These tests all depend upon a particular model or understanding of the carcinogenesis process and interpretation of results is made accordingly. Each of them has its drawbacks and limits of reliability. The possibility of incorporating a number of such short-term tests into a battery for predicting carcinogenicity has been surveyed using a statistical Bayesian approach (Pet-Edwards *et al.*, 1982; 1984). Theoretical procedures like the Hansch quantitative structure-activity relation (QSAR) procedure (Hansch, 1965; 1976; Hansch *et al.*, 1964; 1969; 1979; Singer *et al.*, 1977) invoking

lipophilicity, and the pattern-recognition analysis (PARC) method (Wold, 1976; Chou & Jurs, 1979) have also been employed to attempt screening between active and inactive members of the class, with varying degree of success.

Considering the recent advancements made in the elucidation of the biochemical mechanisms and the molecular biology of *N*-nitroso carcinogenesis, the distinct possibility now arises for screening between active and inactive NOC on a primarily *mechanistic* basis. This may be done by invoking the known or postulated mechanistic steps along the carcinogenesis pathway to trace the molecular fate of the compound in the living system, and thereby deduce whether carcinogenic activity is likely to be manifested or not. Current knowledge concerning the activating and deactivating routes involved in NOC carcinogenesis has been steered along in the main by two key concepts the *ultimate carcinogen* hypothesis (Miller, 1970; Miller & Miller, 1969; 1976; 1977) and the *somatic mutation* theory of carcinogenesis (Boveri 1914; Lowdin, 1977; Ts'O, 1980; Skipper, 1983), as had been reviewed earlier. This chapter seeks to apply the *ultimate carcinogen concept* to propose certain definite *mechanistic* criteria which can help determine whether a given compound would be carcinogenic or not.

III.2. Mechanistic Determinants of Carcinogenic Activity

The specific mechanistic determinants for carcinogenic activity as based upon the ultimate carcinogen (UC) hypothesis could operate at the stage of the parent carcinogen, the proximate carcinogen(s) and the ultimate carcinogen as well. The parent carcinogen for NOC refers to the compound as administered to the living system. The proximate carcinogen species include the α-hyd

roxyalkylnitrosamine or monoalkylnitrosamine (for the indirect-acting *N*-nitrosamines) and the alkanediazohydroxide as well (common to all classes of NOC). These various mechanistic determinants are discussed as follows for the separate classes of direct-acting and indirect-acting NOC and also for determinants which could be common to both :

[A]. Direct-acting N-nitroso carcinogens:

For the direct-acting types of NOC, the sequence of molecular events resulting in formation and action of the UC may be summed up as follows : *(a)* administration of the parent NOC, *(b)* transport to site of tumour induction, *(c)* intracellular lipophilic uptake, *(d)* generation of the proximate and ultimate carcinogen species, and *(e)* attack of DNA by the ultimate carcinogen.

1. Administration and transport routes : The effect of these concerns the parent NOC only. Administration routes and dosage schedules are not without their effects upon the carcinogenic effect of the NOC, *viz.* the tumour induction site, latent induction period and tumour yield, and in some cases may even decide whether the carcinogenic effect would be manifested or not. For instance, *N*-nitroso-*n*-butyl-*N'*-nitroguanidine is non-carcinogenic when administered orally to Wistar rats (Matsukura *et al.*, 1979), but produces subcutis tumours when administered by sc injection (Sano *et al.*, 1978). The transport routes adopted by the NOC depend largely upon the mode of administration, and are of consequence for the site of tumour-induction (Magee, 1976; Preussmann & Stewart, 1984), and are discussed in more detail later in the context of choice of target organ (Chapter VI).

2. Effect of lipophilicity : In the course of the intracellular lipophilic uptake, the lipid bilayer nature of the cell membrane calls for some degree of lipophilicity of the parent NOC. This forms, in part, the basis for the QSAR approach to NOC carcinogenicity (Singer *et al.*, 1977; Hansch, 1979). It was found that lipophilicity constituted a necessary but not sufficient condition for carcinogenic activity, since some very lipophilic NOC are quite inactive as carcinogens. This calls for recourse to other mechanistic factors, as dealt with below, especially in Sec. III.3.

3. Generation of Proximate and Ultimate Carcinogens : The basic chemistry of direct-acting parent NOC would dictate that all direct-acting NOC without exception are capable of generating reactive electrophiles *in vivo*, as described in Chapter II. This would mean that all *N*-nitrosamides are capable of this spontaneous decomposition, except for the cases of *N*-trialkylnitrosoureas and similar compounds which do not yield a facile six-membered transition state for the decomposition and are therefore indirect-acting NOC. If all direct-acting NOC are capable of yielding potential UC species *in vivo*, the question then arises as to why some are carcinogenic and others not so. This question will be dealt with in the paragraph of this section dealing with the mechanistic determinants common to both direct and indirect-acting classes of *N*-nitroso compounds.

[B]. Indirect-acting N*-nitroso carcinogens*

For indirect-acting NOC, the sequence of molecular events culminating in the generation and DNA-modifying role of the UC

may be summed up as follows: *(a)* administration of the parent NOC, *(b)* transport to site of initial metabolism, *(c)* intracellular lipophilic uptake, *(d)* metabolism to the proximate carcinogen form, *(e)* transport to site of UC generation, *(f)* generation of the UC, and *(g)* attack of DNA at critical sites by the ultimate carcinogen species.

1. Administration and transport routes : Unlike some direct-acting NOC, the administration route for indirect-acting NOC apparently has no effect upon determining the presence/absence of carcinogenic activity in any case documentated so far. This may be attributed to the fortituous choice of administration routes employed, or conversely to the possibility that at least some organs reached by the route employed have the potential for undergoing neoplastic changes. It may be stated, however, that the transport of the carcinogen should be such as to reach the site of metabolism (whether intitial or final), which is the liver in many cases. In some cases, the organ of initial metabolism may not be the organ where the carcinogenic effect manifests itself. In such cases, it may be proposed that the initial metabolite produced (the proximate carcinogen) is fairly stable and capable of being transported to the actual site of tumour induction. This apparently seems to be the case for some cases pertaining to organ-specificity of action, and is dealt with further in Chapter VI dealing with organ-specific effects.

2. Role of lipophilicity : As for direct-acting NOC, indirect-acting NOC also have to undergo penetration of the lipid bilayer of the cell wall to reach the organelle where metabolism can

occur. As Singer *et al.* (1977), Hansch, (1979) have discovered, lipophilicity forms an essential but not sufficient determinant for carcinogenic activity here. Recourse has to be made to other mechanistic factors here too.

3. Generation of proximate/ultimate carcinogens :

The ability to generate the reactive potential DNA-modifying agents depends upon two factors : *(a)* the presence of the requisite enzymes for activating metabolism, and *(b)* proneness of the parent NOC itself to undergo activating metabolism (*viz.* α-hydroxylation). These factors, particularly the second one, are dealt with in expanded form in the next section (Sec. III.3).

[C]. Common features for direct- and indirect-acting NOC

The basic common feature for both classes of NOC lies in the role played by the UC to modify DNA. So that major factors, within the context of the UC theory, that pertain to determining of presence/absence of carcinogenicity is whether the UC can exist sufficiently long *in vivo* to be able to reach the critical sites on DNA and modify it. As will be seen in Sec. III.3, scope is open for the UC to be lost through *hydrolysis* or through various *cyclisation* routes, thereby robbing the parent NOC of its potential to modify DNA.

III.3 The Mechanistic Model

Upon accumulating the wealth of experimental and mechanistic findings available for the *in vivo* metabolism and transformation of the parent NOC to its UC form, a mechanistic model may be proposed here as follows to define the mechanistic criteria which help determine whether a given NOC will be carcinogenic or not.

For indirect-acting N-nitrosamines:

Since the criteria of appropriate administration route, effective transport to site of metabolism and lipophilicity (as dealt with in Sec. III.2 above) appear self-evident as necessary for carcinogenicity, this section concerns only the remaining criterion of ability of the parent NOC to be metabolised to create the reactive electrophilic UC species. Assuming that the parent indirect-acting NOC arrives at a suitable site where activating metabolism can take place, the next question is whether it will undergo metabolism or not. In the context of the well-accepted α-hydroxylation pathway for activating metabolism of indirect-acting NOC, the following mechanistic criteria emerge as of significance for the presence/ absence of carcinogenicity.

(a) Susceptibility of α-hydrogens to enzymatic attack:

The cytochrome P_{450} enzymatic α-hydroxylation mechanism points to the importance of the presence, accessibility and reactivity of the *α*-hydrogens for indirect-acting *N*-nitrosamine carcinogenic activity. The mechanistic criteria of relevance here may be defined as follows:

Mechanistic criterion 1 : Presence of α-hydrogens for enzymatic attack:

For any indirect-acting *N*-nitroso compounds to exhibit a carcinogenic or tumorigenic activity, the parent compound must obviously need to possess at least one hydrogen attached to the *α*-carbon, so that the parent compound be capable of undergoing biochemical α-hydroxylation mediated by the metabolising cytochrome P_{450} enzyme system. It is noteworthy that in the list

of Preussmann and Stewart (1984), not a *single* active indirect-acting NOC is known which does not possess at least one α-hydrogen in its structure. The reported negative response of *N*-nitrosodiphenylamine in rats may thus be reasonably attributed to absence of α-hydrogens in this molecule (Cardy, *et al.*, 1979).

Mechanistic criterion 2 : Sufficient reactivity of α-hydrogens

The triplet oxygen of the cytochrome P_{450} enzyme system is believed to have electrophilic character, so that nucleophilicity and negative charge on the α-hydrogens would be a contributing factor to α-hydroxylation facility. Furthermore, the unpaired electron in the SOMO (the singly-occupied MO) calls for frontier-orbital reactivity consideration both from the highest occupied as well as from the lowest empty MO of the nitrosamine which significantly involves the α-hydrogens.

Mechanistic criterion 3. Steric accessibility of α-hydrogens:

Apart from availability and reactivity considerations, the α-hydrogens should obviously be sterically accessible to the action of the cytochrome P_{450} enzyme. The exact steric determinants for achieving a fit between the enzyme and the nitrosamine substrate are not known, but it may be safely proposed that kind of intramolecular steric blocking or shielding of the α-hydrogens from any sort of outside attack would be detrimental to the α-hydroxyl action step (Lijinsky & Taylor, 1975; Okada & Ishidate, 1977; Gold *et al.*, 1981; Lijinsky & Taylor, 1976).

(b) Low competition from deactivating metabolic routes:

The α-hydroxylation pathway being assumed the chief

activation pathway, serious competition from the deactivating β- to ω-hydroxylation routes would proved to be deletorious to the carcinogenic effects of *N*-nitrosamines. This could happen if the alkyl group chain is very long on both sides. One way out of this would be the chain shortening (Knoop-type) degradation pathways of course (Okada & Ishidate, 1976; Suzuki *et al.*, 1981; Singer *et al.*, 1981). In the context of their nonfunctionality or relative lack of availability, the path would be opened for these deactivating hydroxylating routes to exert their depleting effect with the excretion and the loss of the potential alkylating species in the form of ester products like the glucuronide derivatives of the β-to ω-hydroxylation (or oxidation) products (Maher *et al.*, 1968; Marroquin & Coyote, 1970; Irving *et al.*, 1969). This could perhaps help account for the inactivity of dialkylnitrosamines where the number of carbon in both alkyl group exceeds seven (Preussmann & Stewart, 1984). This thereby calls for the following mechanistic criterion for activity :

Mechanistic criterion 4 : At least one alkyl group in a dialkylnitrosamine should not be very long

For direct- and indirect-acting NOC.

For both direct- and indirect-acting NOC, the unifying factor is the role of the UC in modifying DNA. Innate reactivity features of the UC may be conceived here with regard to this inability, since the major groove of DNA is large enough to accomodate the attack of a UC of very large molecular size. All diazonium ions would also be capable of reacting with DNA. Thus, the only possibility for inability to modify DNA would lie

in the *loss* of the UC species. Depending upon the scope for in vivo loss of UC species, the potential ultimate carcinogen species may suffer lesser or greater depletion *in vivo*, owing to the following factors.

(a) Detoxifying role of UC hydrolysis :

All the reactive UC species formed in the biological system would be expected to undergo at least partial loss chiefly due to the action of water (which hydrolyses the alkanediazonium ions to carcinogenically inactive alcohols), or of other cellular biomolecules like thiols (sulfhydryl groups) and other non-DNA nucleophiles. When this deactivating hydrolysis/solvolysis is sufficiently predominant, it could result in effectively complete depletion of the UC before it reaches the DNA target. Factors which contribute to susceptibility towards UC hydrolysis include *high molecular weight* and *large surface area* of the alkanediazonium ion, since these factors would tend to retard the progress of the UC through the cellular medium from its site of formation to the site of DNA modification, thereby allowing for the fast reaction of UC hydrolysis to have greater chance and time for performing its depletion function. Such considerations lead to the next mechanistic criterion for activity.

Mechanistic criteria 5 : The alkyl group in the UC should not be very bulky or heavy.

(a) Role of UC cyclisation :

In some cases, the proximate and/or the ultimate carcinogen species may be prone to undergo an intramolecular cyclisation reactions, yielding products which have no carcinogenic activity.

Such cases are highly individualised, and are dealt with in detail in the next section, but nevertheless can be summarised in the form of the following mechanistic criterion :

Mechanistic criterion 6 : The proximate and ultimate carcinogens should not be lost through cyclisation to carcinogenically inactive products.

III.4 Applications of the Model:

The above mechanistic criteria for carcinogenic activity of NOC are now formulated through quantification into a simple model for screening between active and inactive NOC, which is based upon the ultimate carcinogen theory, calling for the role of the parent, proximate and ultimate carcinogen species. Since the direct-acting NOC possess no mechanistic criterion peculiar to them (which is not the case for indirect-acting NOC), the latter are treated first, followed by the features common to both.

1. Indirect-acting NOC (N-nitrosamines)

Quantification is made of the mechanistic features presented in the previous section for indirect-acting NOC, especially the dialkylnitrosamine.

(a) Presence of α-hydrogens : Mechanistic Criterion 1 above, relevant only for indirect-acting *N*-nitrosamines, is applied here. Tables III.1, III.2 and III.3 represent the effects of the presence or absence of α-hydrogens on the carcinogenic activity of some symmetrical dialkylnitrosamines, asymmetrical dialkylnitrosamines, and cyclic *N*-nitrosamines respectively.

Table III.1 Effects of presence/absence of α-hydrogens and competition factor F_α on presence /absence of carcinogenic activity[a] on symmetrical dialkylnitrosamines (indicated simply by name of alkyl groups).

No.	Alkyl group	Activity	CRITERION 1 P/A	CRITERION 2 F_α	REMARKS 1	REMARKS 2
1.	Methyl	+	P	6/6 (1.000)	✓	✓
2.	Ethyl	+	P	4/10 (0.400)	✓	✓
3.	*n*-propyl	+	P	4/14 (0.286)	✓	✓
4.	Isopropyl	+	P	2/14 (0.143)	✓	✓
5.	*n*-butyl	+	P	4/18 (0.222)	✓	✓
6.	*sec*-butyl	weak	P	2/18 (0.111)	✓	✓
7.	*n*-pentyl	+	P	4/22 (0.182)	✓	✓
8.	*n*-octyl	-	P	4/34 (0.117)	x	✓
9.	cyclohexyl	-	P	2/22 (0.091)	x	✓
10.	Benzyl	-	P	4/14 (0.286)	x	x
11.	Phenyl	-	A	0/10 (0.000)	✓	✓
12.	Allyl	-	P	4/10 (0.400)	x	x
13.	CH_2COOH	-	P	4/4 (1.00.)	x	x
14.	CH_2COCH_3	+	P	4/10 (0.400)	✓	✓
15.	CH_2CF_3	-	P	4/4 (1.000)	x	x
16.	CH_2CH_2CN	-	P	4/8 (0.500)	x	x
Untested cases					*Prediction*	
17.	*tert*-Butyl	?	A	0/18 (0.000)	-	-
18.	2-Pyridyl	?	A	0/10 (0.000)	-	-
19.	8-Adenyl	?	A	0/1 (0.000)	-	-
20.	*n*-Nonyl[b]	?	P	4/38 (0.105)	+	-
21.	*n*-Decyl[b]	?	P	4/42 (0.095)	+	-

a Taken from Preussmann & Stewart (1984)
b Net prediction as negative based on F_α values

Table III.2 Effects of presence/absence of α-hydrogens and competition factor F_α upon presence/absence of carcinogenicity[a] in some asymmetrical dialkylnitrosamines (indicated by alkyl groups R_1 and R_2).

No.	R_1	R_2	activity	*CRITERION* 1 P/A	2 F_α		*Remarks* 1	2
1.	Methyl	Vinyl	+	P	4/5	(0.667)	✓	✓
2.	Ethyl	Vinyl	+	P	3/8	(0.375)	✓	✓
3.	Methyl	*n*-propyl	+	P	6/10	(0.500)	✓	✓
4.	Methyl	Allyl	+	P	5/8	(0.625)	✓	✓
5.	Methyl	Phenyl	+	P	3/8	(0.375)	✓	✓
6.	Ethyl	cyclohexyl	+	P	4/14	(0.286)	✓	✓
7.	Methyl	*n*-octyl	+	P	5/20	(0.250)	✓	✓
8.	Methyl	*n*-Tetradecyl	+	P	5/32	(0.156)	✓	✓
9.	Methyl	Benzyl	+	P	5/10	(0.500)	✓	✓
10.	Methyl	*tert*-Butyl	-	P	3/12	(0.250)	x	x
11.	Ethyl	*tert*-Butyl	-	P	2/14	(0.143)	x	x
12.	*n*-Butyl	*tert*-Butyl	-	P	2/18	(0.091)	x	✓
13.	Methyl	1,1-dimethylbenzyl	-	P	3/14	(0.207)	x	x
14.	CH_2COOH	*n*-Butyl	-	P	4/11	(0.364)	x	x
Untested cases							*Predictions*	
15.	*tert*-Butyl	2-Pyridyl	?	A	0/13	(0.000)	-	-
16.	*tert*-Butyl	Phenyl	?	A	0/14	(0.000)	-	-
17.	Phenyl	2-Pyridyl	?	A	0/9	(0.000)	-	-
18.	Ethyl	*n*-Propyl	?	P	4/12	(0.333)	+	+
19.	Ethyl	Phenyl	?	P	2/10	(0.200)	+	+
20.	*n*-Propyl	Vinyl	?	P	3/10	(0.300)	+	+

[a] Taken from the review of Preussmann & Stewart (1984)

Table III.3 Effects of presence/absence of the α-hydrogens and competition factor Fα on presence/absence of carcinogenic activity[a] in Cyclic N-nitrosamines

No.	Compound Name	Activity[a]	CRITERION 1 P/A	CRITERION 2 F_α	Remarks 1	Remarks 2
1.	N-nitrosoproline	-	P	3/7 (0.428)	x	x
2.	N-nitrosoproline ethyl ester	-	P	3/12(0.250)	x	x
3.	N-nitrosohydroxy proline	-	P	3/6 (0.500)	x	x
4.	N-nitrosoguvacoline*	-*	P	4/10(0.400)	x	x
5.	N-nitrosopipecolic acid*	-*	P	3/9 (0.333)	x	x
6.	N-nitrosoisonipecotic acid*	-*	P	4/9 (0.444)	x	x
7.	N-nitroso-2,6-dimethylpiperidine*	-*	P	2/14(0.143)	x	(x)
8.	N-nitroso-2,2,6,6-tetramethylpiperidine*	-*	A	0/18(0.000)	✓	✓
9.	N-nitrosomethyl phenidate*	-*	P	3/18(0.167)	x	x
10.	N-nitroso-N'-methylpiperazine	-	P	4/11(0.364)	x	x
11.	N-nitroso-pyrrolidine	+	P	4/8 (0.500)	✓	✓
12.	N-nitrosopiperidine	+	P	4/10(0.400)	✓	✓
13.	N-nitroso-3,5-dimethylpiperidine	+	P	4/14(0.286)	✓	✓
14.	N-nitrosohexa methyleneamine	+	P	4/12(0.333)	✓	✓
15.	N-nitroso-octamethyleneamine	+	P	2/16(0.250)	✓	✓
16.	N-nitroso-2,6-dimethylmorpholine	+	P	4/12(0.333)	✓	✓
17.	N-nitroso-N'-3,5-trimethylpiperazine	+	P	4/15(0.267)	✓	✓

[a] Taken from the review of Preussmann and Stewart (1984)
* Negative at dosage administered; may be not maximum tolerated dose

These data include representative tested cases for activity (positive) and inactivity (negative), and also include some cases that have not been tested so far. Note that these tables are not exhaustive from the point of view of rationalising *activity*, since every single one of the 198 *N*-nitrosamines (dialkyl and cyclic) surveyed by Preussman and Stewart (1984) which tested positive for animal carcinogenicity is marked by the presence of at least one α-hydrogen. Emphasis is therefore laid upon some noteworthy cases of tested *inactive* *N*-nitrosamines. The Tables present the identity of the compound, the presence or absence of α-hydrogens (indicated as P and A respectively), the presence or absence of carcinogenic activity (denoted as + and - respectively), and also including a remark (indicated by a tick) on whether the prediction is in line with the expectations of this particular mechanistic criterion of presence/absence of α-hydrogens. For symmetrical dialkylnitrosamines (Table III.1), the compound is identified simply through the alkyl group present in the compound. For asymmetrical dialkylnitrosamines (Table III.2), the compound is identified in terms of the two alkyl groups R_1 and R_2 present in the compound. Cyclic *N*-nitrosamines are simply identified by name in Table III.3.

The α-hydrogen presence/absence criterion (given as Criterion A in Tables III.1 to III.3) serves to successfully predict the reported carcinogenic inactivity of *N*-nitrosodiphenylamine (Druckrey *et al.*, 1967; Cardy *et al* 1979; Argus & Hoch-Ligeti, 1961; Boyland *et al.*, 1968) given in Table III.1, and of *N*-nitroso-2,2,6,6-tetramethylpiperidine (Lijinsky & Taylor, 1975)

in Table III.3. Both these cases may be reasonably attributed to the absence of α-hydrogens in these compounds. Following this criterion, Table III. 1 lists untested symmetrical dialkylnitrosamines (Nos. 17-19) like *N*-nitrosobis(*ter*-butyl)-amine and *N*-nitrosobis(*2*-pyridyl)amine, Table III.2 lists some untested asymmetrical members (those numbered 15-17), and Table III.3 lists members (those numbered which 18-20), predicting them all to be carcinogenically inactive on this same basis.

The α-hydrogen presence/absence criterion obviously does well to predict the carcinogenic activity of each of the 198 active *N*-nitrosamines possessing α-hydrogens, but fails to explain the inactivity of a large number of *N*-nitrosamines which also possess α-hydrogens. These include those numbered as 10, 12, 13, 15 and 16 in Table III.1, those numbered as 10, 11, 12, 13 and 14 in Table III.2, and those numbered as 1 to 7 with 9 and 10 in Table III.3. Obviously, this criterion is not sufficient in itself to screen for inactivity, and has to be applied in conjunction with others, as will be seen as follows in this and the next Chapter.

(b) Sufficient reactivity of α-hydrogens : Even if there are α-hydrogens present, a large number of cases present themselves as inactive. The next step would be to invoke the Mechanistic Criterion 2 above (not the same as Criterion B of Tables III.1 to III.3) of sufficient reactivity of the α-hydrogens for enzymatic radical abstraction followed by formation of the α-hydroxylated product. The facility of α-hydrogens towards the hydrogen-abstraction step may be expressed in terms of calculated indices, namely, the Mulliken charges Q_h on α-hydrogens, the Wiberg bond

Table III.4 Effects of α-hydrogens reactivity (towards electrophilic triplet oxygen) upon presence/absence of carcinogenic activity in dialkylnitrosamines

No.	Compound	Activity	Q_h	T_{fo}	W_{ch}	Remarks
1.	N-nitrosoethyl(2,2,2-triflouroethyl)amine	+	0.098	1.521	0.955	follows the expected trend
2.	N-nitrosobis(2,2,2-triflouroethyl)amine	-	0.091	1.685	0.957	follows the expected trend
3.	N-nitrosoiminodi acetic acid	-	0.087	1.702	0.955	against the expected trend
4.	N-nitrososarcosine	+	0.097	2.202	0.958	follows the expected trend
5.	N-nitrosobis(2-cyanoethyl)amine	-	0.081	1.503	0.956	against the expected trend
6.	N-nitrosodicyano methylamine	+	0.090	2.068	0.955	follows the expected trend
7.	N-nitrosodiallyl amine	-	0.086	1.637	0.957	follows the expected trend
8.	N-nitrosodi-n-propylamine	+	0.095	5.021	0.956	follows the expected trend
9.	N-nitrosobis(2-ethoxyethyl)amine	+	0.090	0.015	0.956	follows the expected trend
10.	N-nitrosobis(2-diethoxyethyl)amine	-	0.082	0.027	0.957	follows the expected trend

Table III.4 continuation

11. N-nitroso-n-propyl(2-hydroxypropyl)amine	+	0.090	9.721	0.955	follows the expected trend
12. N-nitrosoethyl(2-hydroxypropyl)amine	-	0.086	4.071	0.958	against the expected trend
13. N-nitroso-n-butyl(3-carboxypropyl)amine	+	0.092	0.011	0.953	follows the expected trend
14. N-nitroso-n-butyl(2-carboxyethyl)amine	-	0.088	1.517	0.958	against the expected trend
15. N-nitroso-O-N-diethylhydroxylamine	+	0.093	8.121	0.955	follows the expected trend
16. N-nitroso-O-N-dimethylhydroxylamine	-	0.088	2.124	0.958	against the expected trend
17. N-nitroso-N-methyl-2-aminopyridine	+	0.090	7.179	0.957	follows the expected trend
18. N-nitroso-N-methyl-3-aminopyridine	-	0.089	7.383	0.956	against the expected trend
19. N-nitrosodiphenyl amine	-	0.098	5.203	0.952	follows the expected trend
20. N-nitrosodioctyl amine	-	0.089	5.784	0.954	follows the expected trend

strength index W_{ch} for the C-H bond broken, and the frontier orbital factor T_{fo} given by:

$$T_{fo} = C_h/(E_s-E_h) + C_l(E_l-E_s) \qquad \text{(III.1)}$$

where, C_h and C_l are respectively the HOMO and LEMO coefficients of the α-hydrogen 1_s orbital, E_h and E_l are the HOMO and LEMO energy levels respectively, E_s being the energy level of the singly occupied molecular orbital of the triplet oxygen. The term T_{fo} is a factor expressed for the frontier orbital interaction energy between the two systems. Table III.4 presents the results of model gas-phase AM_1 SCF molecular orbital calculations on α-hydrogen reactivity towards the triplet oxygen of the oxidase enzyme for twenty different active and inactive NOC compounds.

One factor contributing to the inactivity noted for *N*-bis(2-trifluoroethyl) nitrosamine (Preussmann *et al.*, 1981) could be the inductive effect of the fluorines upon the nucleophilicity of the α-hydrogens, serving to diminish the same, and thereby making them less reactive towards the electrophilic triplet oxygen of the enzyme. Similarly, *N*-nitrosamines exhibiting strong neighbouring effects from electron withdrawing groups on the α-carbon (like the -COOH and similar groups) could also involve induced reduction in the nucleophilicity of the α-hydrogens of the N-nitrosamines. A detoxifying cyclisation mechanism to rationalise the inactivity of NOC with α-carboxylic acid groups is also given later in this section.

(c) Competition between activating and deactivating enzymatic transformations : Following Mechanistic Criterion 4 above, the competition between α-hydroxylation and β- to *w*-hydroxylation is

expressed here as the competition factor F_α representing the probability of the former occuring in the face of the latter, and given by:

$$F_\alpha = N_\alpha / N_t \qquad \text{(III.2)}$$

where N_α represents the number of α-hydrogens in the nitrosamine, and N_t the total number of *C*-hydrogens in the nitrosamine. Going back to Tables III.1, III.2 and III.3, these present the calculated values of this competition factor F_α for symmetrical dialkylnitrosamines, asymmetrical dialkylnitrosamines and cyclic nitrosamines respectively. It is seen that this quantified criterion (identified in Tables III.1 to III.3 as Criterion B) suffices to correctly predict the inactivity of symmetrical members in Table III.1 like di-*n*-octyl- and dicyclohexyl-nitrosamines, if one takes a value of F_α below 0.12 or so as the cut-off point for prediction of activity. This criterion (taken together with the other criterion of α-hydrogen presence/absence) does not work for those numbered 10, 12, 13, 15 and 16, for which other rationalisations have to be afforded, to be seen later.

2. For Both N-nitrosamines and N-nitrosamides

The factors operating for both direct and indirect-acting types of NOC would obviously have to do with the features of their carcinogenesis mechanisms common to both, *viz.* the stage of generation of the ultimate carcinogen (the alkanediazonium ion). For most direct-acting *N*-nitrosamides, there is no scope for inability to generate a potential alkylating species, since they

all decompose *in vivo*. As we have seen, such an inability in the case of indirect-acting NOC could arise from failure to undergo α-hydroxylation. This sub-section assumes that all cases treated here are capable of generating the UC species *in vivo*. The mechanistic criteria dealt with here for presence of absence of carcinogenic activity thus deal with the UC species through its scope for detoxification.

(a) Scope for in vivo *loss of UC species :-*

The potential UC species may suffer greater or less depletion *in vivo* owing to the following factors :

(i) Hydrolysis of the UC

All UC species undergo at least partial loss chiefly due to the action of water, which hydrolyses the alkanediazonium ion to the alcohol,or of other intracellular agents like thiols (sulfhydryl groups) and other non-DNA nucleophiles (Neal, *et al.* 1978 & 1981; Lotlikar, *et al.* 1980). Since the alkanediazonium ion is extremely reactive and unstable, it would have to be generated at a site very close to the critical target on DNA. Assuming on a statistical basis a certain average distance exists between the site of UC formation and the site of critical DNA modification, it follows that the speed of transit between the two sites would be of consequence for the concentration of the UC species at the moment of DNA modification. This speed may be expected to follow the Graham diffusion law, where the speed V_{uc} is inversely proportional to the square root of the molecular weight M_{dz} of the diazonium ion, so that

$$V_{uc} \propto 1/M_{dz} \qquad \text{(III.5)}$$

$$\text{or} \qquad V_{uc} = p.R_{dz} \qquad \text{(III.6)}$$

where R_{dz} equal $10/M_{dz}$, p being a simple proportionality constant. The numerator 10 in calculating R_{dz} is simply used for convenience in the scale of values of the term R_{dz}, to which the speed V_{uc} is directly proportional. As M_{dz} increases, a limit may be expected to be reached where V_{uc} and R_{dz} drop below a critical value of no further consequence for the carcinogenic effect. This is because the speed V_{uc} would determine the time spent in transit, which again influences the degree of depletion of the UC through hydrolysis (assumed here to be a pseudo-unimolecular reaction). In other words, the heavier the UC is, more chance it has for being depleted in transit.

e that there does exist a subtle difference between direct-ndirect-acting NOC with regard to the detoxifying role of UC ysis for carcinogenicity. This is because for the former, ere is every chance for the direct-acting NOC to decompose right next to the site for critical DNA modification, in which case the UC need not suffer hydrolytic depletion. This is not so for the indirect-acting NOC, for which the site of UC generation (at the microsomes) would not generally be the same as the site of DNA modification (within the cell nucleus). In other words, the effect of UC hydrolytic depletion upon carcinogenicity may be expected to be more pronounced for indirect-acting NOC than for direct-acting NOC. This means that the effect of molecular weight of the UC upon carcinogenicity would be more noteworthy for *N*-nitrosamines than for *N*-nitrosamides.

Table III.5 Effects of proximate carcinogen and ultimate carcinogen molecular weight upon carcinogenicity of dialkylnitrosamines.

No.	Compound	Activity	M_{oh}	R_{oh}	M_{dz}	R_{dz}	R_2	R_1
1.	N-nitrosodimethylamine	+	74.23	1.16	43.05	1.52	✓	✓
2.	N-nitrosodiethylamine	+	102.08	0.99	57.08	1.32	✓	✓
3.	N-nitrosodi-n-propylamine	+	130.05	0.87	71.09	1.19	✓	✓
4.	N-nitrosodiallylamine	-	126.10	0.89	69.11	1.20	x	x
5.	N-nitrosodi-n-butylamine	+	158.24	0.79	85.12	1.08	✓	✓
6.	N-nitroso-N-n-butyl-tert-butylamine	-	158.24	0.79	85.12	1.08	✓	✓
7.	N-nitrosodi-n-octylamine	-	270.30	0.61	140.99	0.88	✓	✓
8.	N-nitroso-N-methy-n-octylamine	+	172.12	0.76	43.05	1.52	✓	✓
9.	N-nitroso-N-di cyclohexylamine	-	210.08	0.69	111.56	0.95	✓	✓
10.	N-nitrosomethyl cyclohexylamine	+	142.20	0.84	43.05	1.52	✓	✓
11.	N-nitroso-N-methylbenzyl amine	+	150.11	0.82	43.05	1.52	✓	✓

12.	N-nitrosodi benzylamine	-	226.40	0.66	119.14	0.92	✓ ✓
13.	N-nitrosomethyl phenylamine	+	136.21	0.86	43.05	1.52	✓ ✓
14.	N-nitrosodi-phenylamine	-	198.78	0.71	105.03	0.98	✓ ✓
15.	N-nitroso-N-methyl-4-amino-azobenzaldehyde	-	164.10	0.78	133.10	0.87	✓ ✓
16.	N-nitroso-N-methyl-4-amino-azobenzene	-	240.31	0.65	209.05	0.69	✓ ✓
17.	N-nitrosomethyl -tetradecyl amine	+	265.14	0.62	43.05	1.52	✓ x
18.	N-nitroso-N-phenylbenzyl amine	+	212.09	0.69	105.03	0.98	✓ x
19.	N-nitroso-N-methyladenosine	+	308.06	0.57	43.05	1.52	| x
20.	N-nitrosodi-n-nonylamine	-	298.32	0.58	155.10	0.80	✓ ✓

Table III.6 Effects of proximate carcinogen and ultimate carcinogen molecular weight upon carcinogenicity of cyclic nitrosamines.

No.	Compound	Activity	M_{oh}	R_{oh}	M_{dz}	R_{dz}	R_2	R_1
1.	N-nitrosoproline	-	144.31	0.83	128.07	0.88	✓	✓
2.	N-nitroso-4-hydroxypiperidine	+	129.20	0.88	113.46	0.94	✓	✓
3.	N-nitroso-4-cyclohexylpiperidine*	-*	196.70	0.71	180.24	0.74	✓	✓
4.	N-nitrosomethylphenidate*	-*	246.30	0.64	230.25	0.66	✓	✓
5.	N-nitroso-N-acetyl-3,5-dimethylpiperazine	+	185.41	0.73	169.11	0.77	✓	✓
6.	N-nitroso-N'-benzoyl-3,5-dimethylpiperazine	+	247.03	0.64	231.19	0.66	✓	✓
7.	N-nitrosoguvacoline*	-*	170.11	0.77	154.08	0.81	✓	✓
8.	N-nitroso-3-hydroxypyrrolidine	+	116.08	0.93	100.12	0.99	✓	✓
9.	N-nitrosonornicotine	+	177.06	0.75	161.14	0.79	✓	✓
10.	N-nitrosopipecolic acid*	-*	158.24	0.79	142.20	0.84	✓	✓
11.	N-nitrosoisonipecotic acid*	-*	158.24	0.79	142.20	0.84	✓	✓
12.	N-nitroso-2,6-dimethylpiperidine*	-*	142.20	0.84	126.10	0.89	✓	✓
13.	N-nitroso-3,5-dimethylpiperidine	+	142.20	0.84	126.10	0.89	✓	✓
14.	N-nitrosomorpholine	+	116.08	0.93	100.12	0.99	✓	✓

Table III.7 Effects of proximate carcinogen and ultimate carcinogen molecular weight upon carcinogenicity of direct-acting N-nitrosamides

No.	Compound	Activity	M_{oh}	R_{oh}	M_{dz}	R_{dz}	R_2	R_1
1.	N-nitroso-N-N'-dimethylurea	+	117.09	0.92	86.01	1.08	✓	✓
2.	N-nitroso-N-allylurea	-	129.20	0.88	68.99	1.20	✓	✓
3.	N-nitroso-N-(2-chloroethyl-urethane	+	179.21	0.75	89.98	1.05	✓	✓
4.	N-nitroso-N-methylacetamide	+	102.08	0.99	71.09	1.19	✓	✓
5.	N-nitroso-5-methylpyrro-lidone	-	130.05	0.88	114.07	0.94	x	✓
6.	N-nitroso-N-ethyl-N'-nitro guanidine	+	161.14	0.79	115.99	0.93	✓	✓
7.	N-nitroso-N-n-pentyl-N-nitro-guanidine	-*	203.06	0.70	115.99	0.93	x	x
8.	N-nitroso-N-n-propyl-N-nitro-guanidine	+	175.10	0.76	115.99	0.93	✓	✓
9.	N-nitroso-N-n-hexyl-N'-nitro-guanidine	-	217.07	0.68	112.99	0.94	✓	✓
10.	N-nitroso-N-isobutyl-N-N'-nitroguanidine	-	189.30	0.73	85.12	1.08	✓	✓

Tables III.5, III.6 and III.7 present data on the effects of proximate carcinogen and ultimate carcinogen molecular weight upon carcinogenicity of dialkylnitrosamines, cyclic nitrosamines and various *N*-nitrosamides. In order to cover the possibility of molecular weight effects upon hydrolysis operating even before the UC stage, *viz* at the proximate carcinogen stage, the molecular weight M_{oh} of the proximate carcinogenic α-hydroxy metabolite of dialkyl and cyclic nitrosamines (together with the corresponding term R_{oh} equal to 10/ M_{oh}) are also calculated and presented in Tables III.5 and III.6. In Table III.7, for the *N*-nitrosamides, the immediate precursor of the UC is the metastable parent carcinogen itself, for which the corresponding molecular weight terms are given as M_{pc} and R_{pc}.

(ii) Detoxification through cyclisation reactions.

In principle,*N*-nitroso compounds are biologically transformed to its reactive species like alkanediazonium ion or alkylcation through enzyme-mediated *α*-hydroxylation (Miller & Miller, 1971; 1981; Singer & Grunberger, 1983; Dipple *et al.*, 1985; Druckrey *et al.*, 1967) or spontaneous decomposition (Druckrey *et al.*, 1967). These reactive species ultimate carcinogens) then act as alkylating agents, which attack and modify the nuclear DNA at the critical sites (Margison & Khleihues, 1975; Abbott & Saffhill, 1977; Richardson *et al.*, 1983; Bhanot & Ray 1986; Preston *et al.*, 1986; Singer, 1975; Pegg, 1977) to initiate and bring about tumour induction.

In some cases, the proximate or ultimate carcinogen species may be prone to undergo further cyclisation reactions, yielding

products which have no carcinogenic activity.

To rationalise this criteria for validity, an eventual cyclisation reaction mechanism for some inactive *N*-nitroso compounds are worked it out to represent this criteria of being inactive. Hopefully, these cyclisation reaction products like the β-lactam, dioxozole, parazole, and mercapto-thiol derivatives are noncarcinogenic products.

Figure.III.8a, portrays the mechanism of cyclisation reaction of *N*-nitrosobis(2-cyanoethyl)amine, a symmetrical dialkylnitrosamine. This compound was tested for carcinogenicity after oral administration on rat species by Lijinsky *et al.*, (1980), and found to be inactive. The reason for being inactive of this compound on test animal may be that the ultimate carcinogen species so formed, might have been proned to undergo a cyclisation reaction to yield a β-lactam product as portrayed in figure III.8*a*, which may not have carcinogenic activity. β-lactam is one of the prime products of ring cleavage cyclisation reaction and is a strong and important stimulating anti-biotics (Bumgardner *et al.*, 1961). The other explanation for this inactive compound may be that the presence of cyano-group on the β-carbon abolishes carcinogenic activity due to inductive effect on the α-carcbon, which may be electronic property (Garcia & Lijinsky, 1973).

Figure III.8*b*, describes the mechanism of activation and cyclisation reaction of *N*-nitrosodiallylamine which has no carcinogenic activity on test animal (rat) after oral administration (Druckrey *et al.*, 1967). The arguement and explanation of its

N-Nitrosobis(2-Cyanoethyl) amine

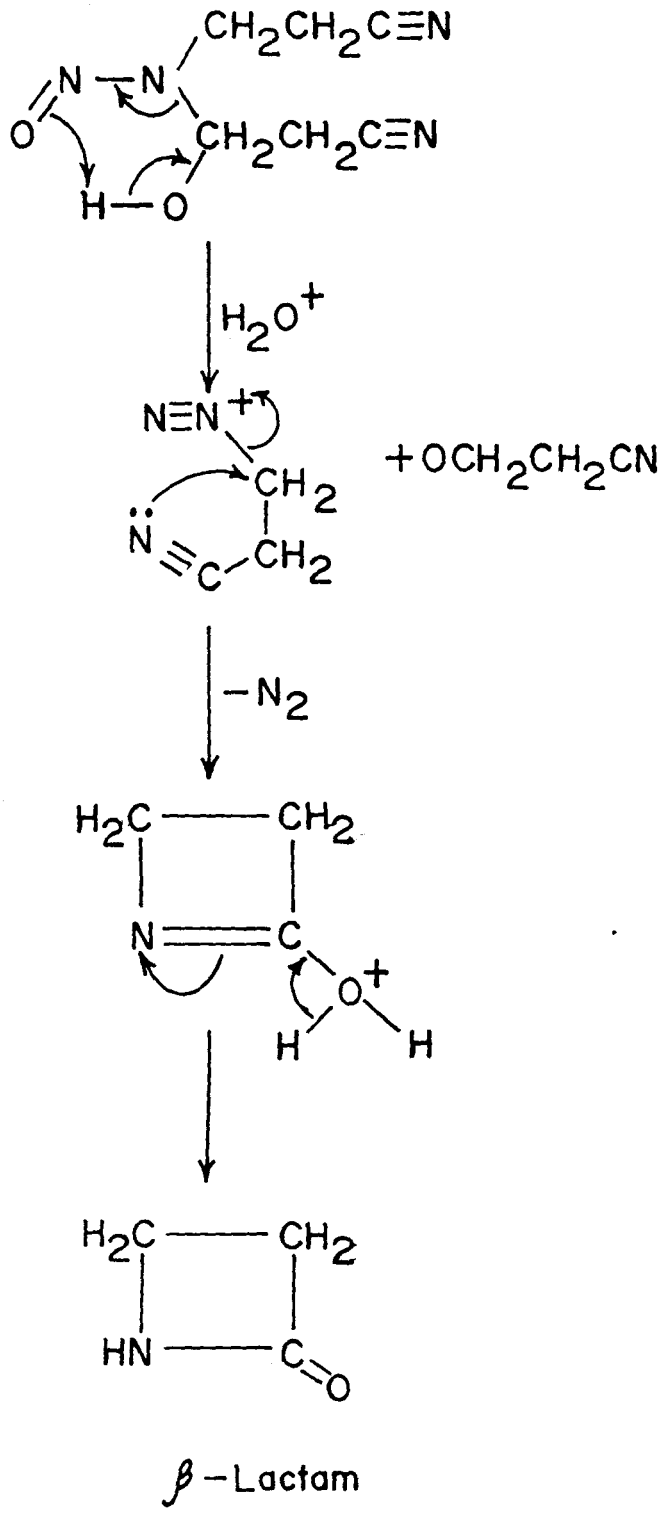
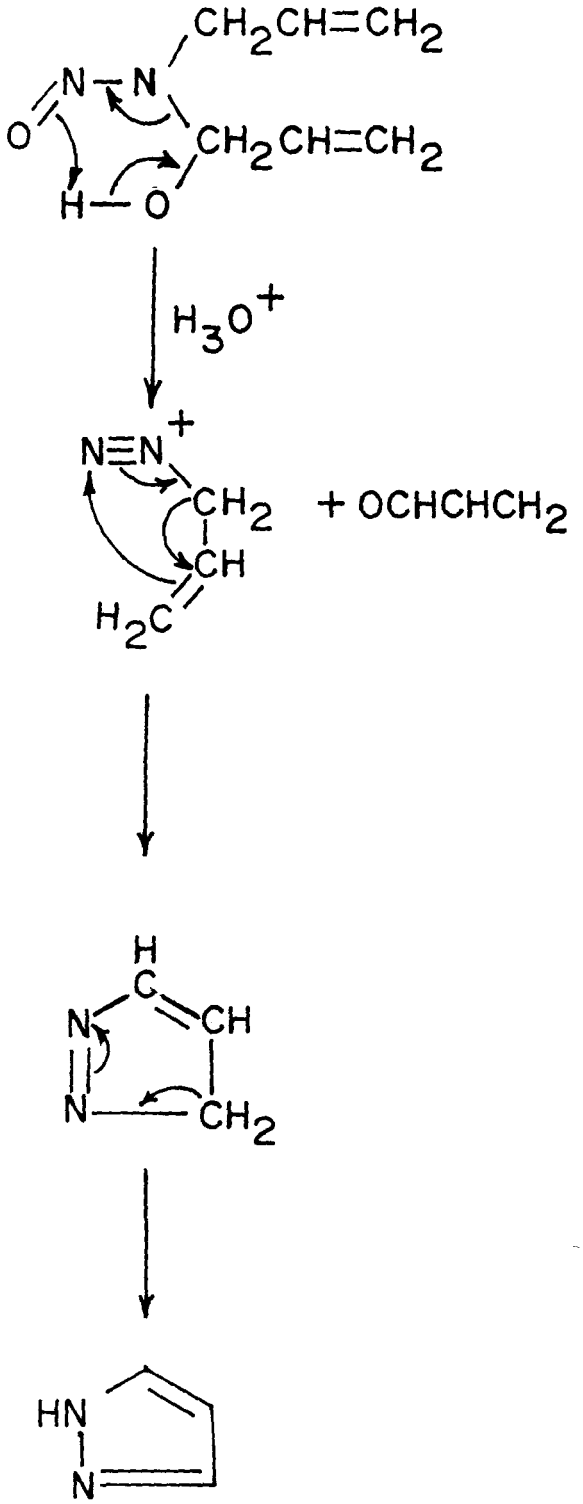


Fig III.8a: Ultimate Carcinogen species undergo a cyclisation reaction to form β-Lactam

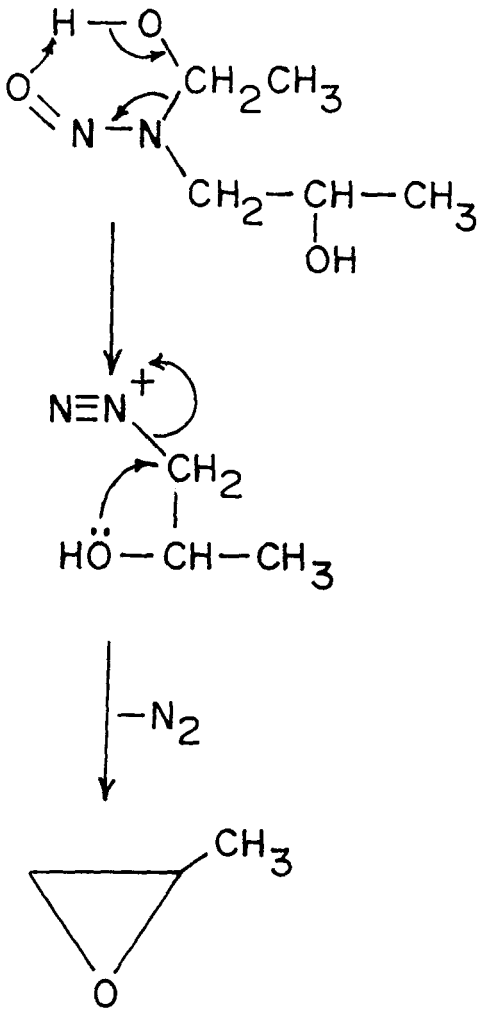
N-Nitrosodiallylamine



Parazole

Fig III-8b: Ultimate carcinogen species undergoing a cyclisation reaction in vivo to yield Parazole.

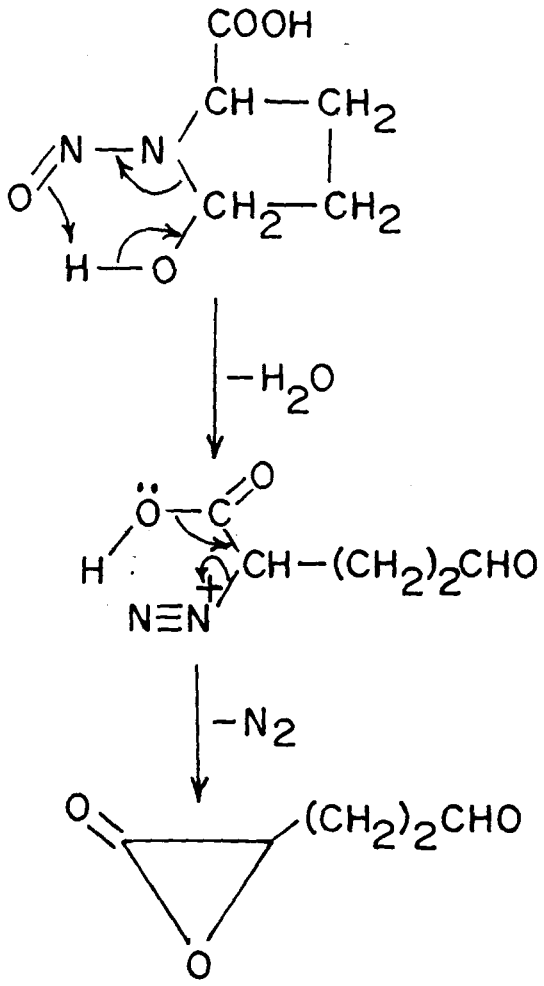
N-Nitroso-N-ethyl
(2-hydroxypropyl) amine



Epoxide

(Fig III.8c): Alkane diazonium ion undergo a cyclisation reaction to yield an Epoxide.

N-Nitrosoproline



Dioxozole

Fig III-8d: Alkanediazonium ion undergoing a cyclisation reaction to yield Dioxozole product.

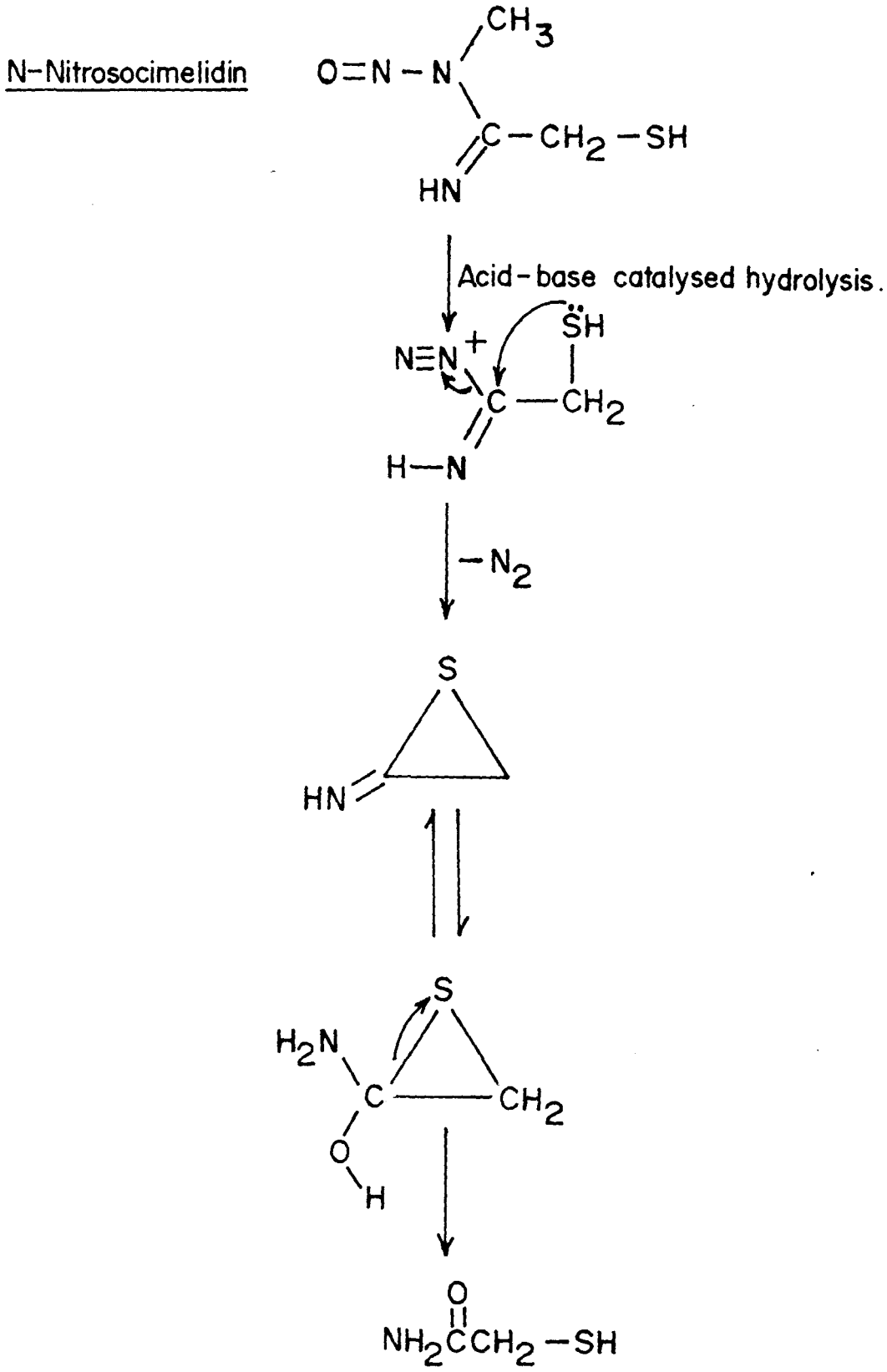


Fig III.8e: The diazonium ion of direct-acting nitrosamides under going cyclisation reaction to yield *Mercapto-thiol*

inactivity could be that immediately, after the formation of the reactive ultimate carcinogen species, undergo further cyclisation reaction to yield a non-carcinogenic product such as parazole derivatives.

Figure III.8*c*, we have an example of an asymmetrical dialkyl-nitrosamine, which is also found inactive in test animal after oral administration (Okada *et al.*, 1977). The likely reason for its being inactive may be also that the reactive uc species is proned to undergo a cyclisation reaction to yield a product like epoxide derivatives, which has no carcinogenic activity.

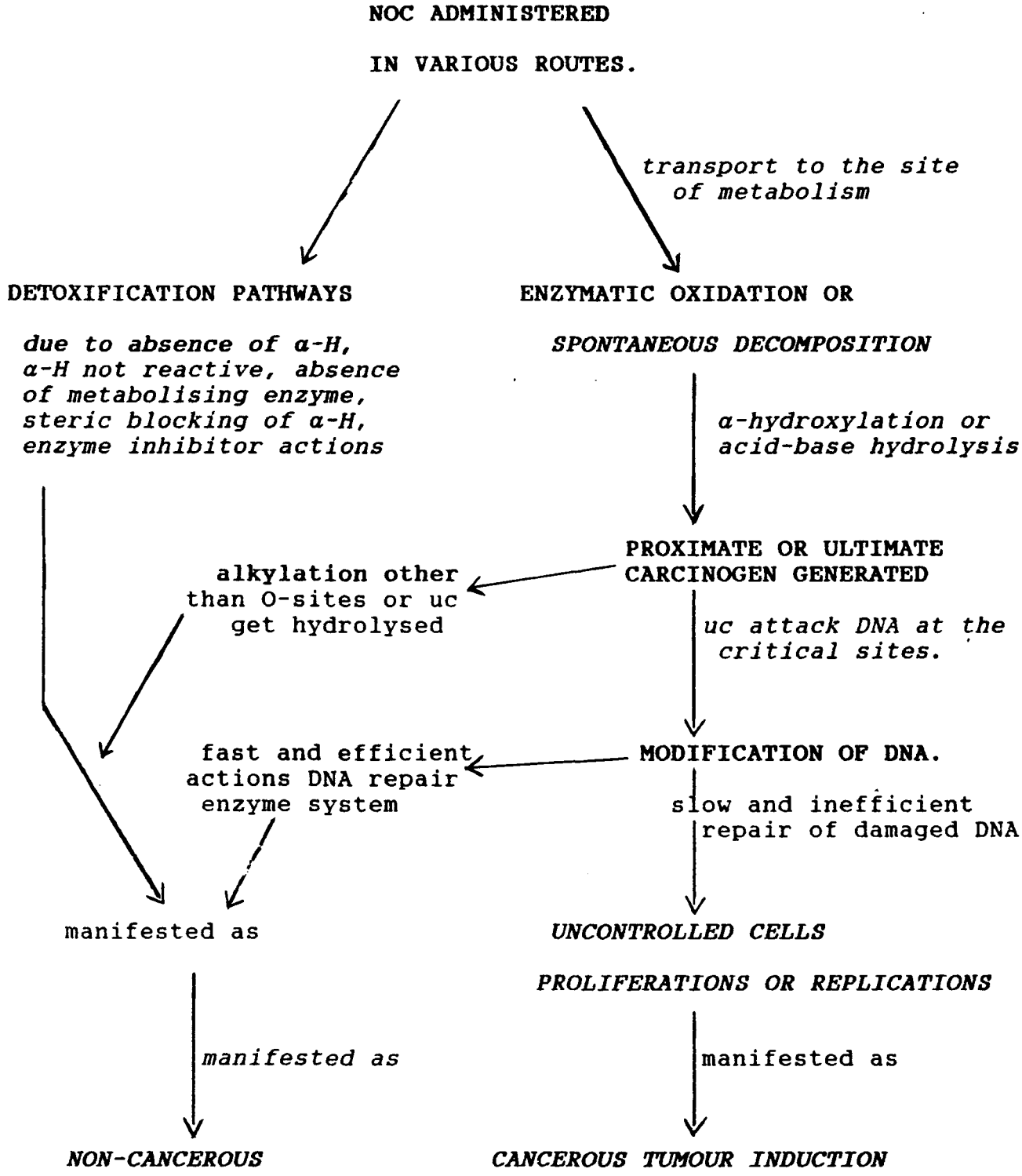
Figure III.8*d*, represents the mechanism of activation and cyclisation reaction of a cyclic nitrosamines exemplified by *N*-nitrosoproline which is also non-carcinogenic in test animal after oral administration in mouse and rats (Greenblatt & Lijinsky, 1972; Garcia & Lijinsky, 1973). Here *N*-nitrosoproline undergo an enzymatic α-hydroxylation to yield the proximate carcinogen which upon dehydration by intramolecular proton transfer, yield the alkanediazonium ion as the ultimate carcinogen. This uc immediately undergo a cyclisation reaction to yield dioxozole derivatives by removal of nitrogen.

Figure III.8*e*, represents the mechanism of activation to uc and its eventual cyclisation reaction of direct-acting *N*-nitroso-cimelidin, which is also inactive after oral administration on different species and strains (Lijinsky *et al.*, 1982; Habs*et al*,. 1982). This compound undergo a spontaneous decomposition or acid-base catalysed hydrolysis to yield the reactive alkanediazonium ion as ultimate carcinogen. Upon removal of nitrogen leads the uc to undergo spontaneous cyclisation reaction to form an inactive

Table III.9: Screening, reasonings, and predictions for the inactivity of N-nitroso compounds, based on the heuristic mechanistic criteria.

Compound Name	Reasons for inactivity
1. N-nitrosobis (2,2,2-trifluoroethyl)amine	α-hydrogens are not reactive enough
2. N-nitrosobis (2-diethoxyethyl)amine	α-hydrogens are not reactive enough
3. N-nitroso (2-cyanoethyl)amine	presence of cyano group at β-carbon inhibits or possible cyclisation reaction of uc to inactive products
4. N-nitrosoiminodiacetic acid	α-hydrogens are not reactive enough
5. N-nitrosdi-*n*-octylamine	due to a high Mol.Wt. of uc species
6. N-nitrosodiphenylamine	due to the absence of α-hydrogens
7. N-nitroso-N-methyl-*tert*-butylamine	conformational barriers due to some steric factors.
8. N-nitroso-N-acetoxymethyl-*tert*-butylamine	conformational barriers due to some steric factors.
9. N-nitroso-N-methyl-(1,1-dimethylbenzene)amine	conformational barriers due to some steric factors.
10. N-nitroso-N-methyl-4-aminobenzylaldehyde	possible cyclisation reaction of uc to some inactive products, also due a high Mol. Wt. of uc species.
11. N-nitroso-N-methyl-4-aminobenzene	due to a high Mol.Wt. of uc species
12. N-nitroso-N-(carboxymethyl)*n*-butylamine	due to functional group effects and possible cyclisation reaction of uc to some inactive products.
13. N-nitroso-2,2,6,6-tetramethylpiperidine	due to the absence of α-hydrogens
14. N-nitrosoproline	due to functional group effects and possible cyclisation reaction of uc to inactive products.
15. N-nitrosocimelidin	due to a cyclisation reaction of uc species to inactive products.

Figure III.10: The overall summation of mechanistic criteria for screening the presence or absence of carcinogenic activity of N-nitroso compounds.



products like mercapto-thiol, that does not have any significant carcinogenic activity.

And finally, Figure III.9 and Figure III.10, represent the overall screening, reasonings, and predictions for inactivity of NOC, and the mechanistic criterions summation for the presence and absence of carcinogenic activity of *N*-nitroso compounds. The application of all these heuristic criteria work well within our framed mechanistic model, in assigning the probable reasons and predictions for inactivity of *N*-nitroso compounds.

The above criteria still do not afford explanation for the lack of carcinogenic activity in NOC containing even one *ter*-alkyl group. For this, recourse has to be made to the role of modified DNA as will be done in the preceeding chapter.

III.5 Concluding Remarks.

All in all, we see that ultimate carcinogen generation in presence of the appropriate metabolising enzymes is favoured by a good intracellular lipophilic uptake, also marked by the presence and sufficient reactivity of α-hydrogens. It may also be mentioned here that for a given compound to be carcinogenic, the rate of activating pathways should dominate over the rate of deactivating pathways in their metabolism. If the repairing enzyme are very efficient and prompt in their repairing action on modified DNA, the parent compound may be found to be functionally non-carcinogenic in expression. As such it will be deemed fit to emphasise that; in the case of active compounds, the action of the DNA repair enzymes are very slow and not prompt enough to repair the damaged DNA efficiently.

CHAPTER FOUR

MECHANISTIC MODEL FOR SCREENING CARCINOGENIC ACTIVITY INACTIVITY OF N-NITROSO COMPOUNDS. ROLE OF MODIFIED DNA

CONTENT

IV.1: INTRODUCTION

The somatic mutation theory of cancer, conceived in germinal form as long back as 1914 by Jena (Boveri, 1914), and put forward more explicitly later (Lowdin, 1977; kipper, 1983; Ts'o, 1980), proposes that the initiation and maintenance of the cancerous state of the cell is intimately involved with mutation

of the nuclear DNA of somatic cells.The discovery of the role and functioning of "cancer genes" the various families of oncogenes (viral and cellular) (Teich, 1986), lends immediate support to this idea. Induction of the appropriate mutations of DNA at critical sites or in a critical manner paves the way for the induction of cancer owing to the procarcinogenic role of the oncoproteins encoded by the modified DNA (Waterfield, 1986; Varmus, 1985). Mechanisms for activation of the latent proto-oncogenes include chromosomal rearrangement or translocation, gene amplification, and point mutations (Teich, 1986; Varmus, 1984; Lawley, 1976). The oncoproteinic products of oncogene expression include kinases, DNA replication switches and epidermal growth factors (Baltimore, 1989; Bishop & Varmus 1984).

The concepts of the somatic mutation theory apply themselves readily in a particularly lucid manner to the phenomenon of carconogenesis by *N*-nitroso compounds (NOC) and alkylating agents (Hathway & Kolar 1980). The modification of DNA by these classes of compounds has been studied extensively experimentally as well as theoretically (Parada & Weinberg, 1983; Shah *et al.*, 1981; Sukumar *et al.*, 1984; Balmain & Progvell, 1983; Eva & Aaronson, 1983; Hirani-Hojatti *et al.*, 1987). NOC and alkylating agents are well-known mutagens as a class (Magee & Barnes, 1970; Harris, 1976; Lijinsky *et al.*, 1976; Lijinsky *et al.*, 1983; Lijinsky *et al.*, 1981; Prival & Mitchell, 1983), and good correlations have been observed between their carcinogenic and mutagenic effects (de Vos *et al.*, 1988; Baltimore, 1989). The fact that these compounds are known to induce point mutations (Lawley, 1976 &

1984; Varmus, 1984; Osborne, 1984), including base substitutions (Bargmann *et al.*, 1986; Bargmann & Weinberg, 1988), is quite significant in view of the observation that point mutation is a simple and well-documentated mechanistic basis for conversion of the latent proto-oncogenic sequence to the carcinogenically active one, as has been observed for the *ras* and *neu* types of oncogenes (Varmus, 1984; Bargmannet *al.*, 1986; 1988; Rasheed *et al.*, 1983). Carcinogenesis by methylnitrosourea in mice has been directly linked to point mutational activation of an oncogene of the *N-ras* family (Taparowsky *et al.*, 1983; Tainsky *et al.*, 1984). The *ras* oncogene has also been shown to be activated from its latent to its active form by reaction with methylnitrosourea *in vitro* (Varmus, 1984).

The importance of point mutations serving as a viable basis for oncogene activation by NOC and alkylating agents is further heightened by the quantum of evidence concerning the base-pairing properties of DNA base residues alkylated by these compounds. Out of the 16 odd DNA sites known to be alkylated by these compounds, the O^6-guanine and O^4-thymine sites emerge as having special significance for point mutations (Singer *et al.*, 1986; Abbott & Saffhill, 1978). *In vitro* and *in vivo* results have been obtained which demonstrate the ability of these modified bases to mispair with noncomplementary bases when incorporated into templates for nucleic acid polymerases, where O^6-alkylguanine can pair like adenine (Mehta & Ludlum, 1978; Abbott & Saffhill, 1979; Bhanot, 1986) and O^4-alkylthymine like cytosine (Singer *et al.*, 1978; Preston *et al.*, 1986), leading to the *GC-->AT* and *TA-->CG* transitions respectively. This is in contrast to the behaviour of

N^7-alkylguanine (the chief alkylation product) which does not mispair (Schoental, 1969; Ludlum, 1970; Scriber & Ford, 1982) in a promutagenic fashion, behaving like unalkylated guanine. This promutagenic role of the *O*-alkylated bases is complemented by their likely procarcinogenic role as well, where correlations have been described between tumour incidence and persistence of these *O*-alkylated bases in the tissues of rodents treated with methylating carcinogens (Loveless, 1969; Loveless & Hampton, 1969; Gerchman & Ludlum, 1978).

IV.2 Role of Modified DNA for N-nitroso Carcinogenesis

The role of modified (especially alkylated) DNA for *N*-nitroso carcinogenesis and carcinogenesis as a whole has been dealt with in detail in sections II.3 & I.4. The following observation serve to highlight the connection between DNA modification and the *N*-nitroso carcinogenesis.

It is well known that *N*-nitroso compounds modify the nuclear DNA of somatic cells, besides other macromolecules like proteins, as shown by *in vitro* and *in vivo* studies (Pegg, 1977; Lawley, 1978; Bartsch *et al.*, 1979; Beranek *et al.*, 1980; Singer *et al.*, 1978; Richardson *et al.*, 1987). They also act as genotoxic agents (Rao *et al.*, 1984) and have been observed to induce point mutations (Essigman *et al.*, 1986). Their ability to induce point mutations is of particular significance for carcinogenesis when considered in the light of the impressive array of evidence for point mutations serving as a mechanism for the activation of proto-oncogenes to their carcinogenically active counterparts, as has been noted for oncogenes of the *ras* and *neu* families.

Furthermore, there is evidence, both *in vitro* and *in vivo*, for the ability of NOC to convert proto-oncogenes to their active forms (Varmus, 1984; Hirani-Hojatti *et al.*, 1987; Sukumar *et al.*, 1983). The nature of these activating point mutations has been shown to be of a base-substitutional kind (*ie.* either transitions or tranversions).

The carcinogenic role of modified DNA for *N*-nitroso carcinogenesis (as well as other alkylating agents) is highlighted at the simple molecular level by observations concerning the template properties of nucleic acid bases modified through alkylation. The 16 odd products of *in vitro* and *in vivo* alkylation of DNA by NOC include the products of alkylation at the O^2-T, O^4-T and O^6-G positions. These *O*-alkylated bases, unlike many other instances of *N*-alkylated bases, demonstrate the property of incorporating the wrong base during DNA synthesis (or action of nucleic acid polymerases upon substrates containing these modified bases). This could in principal lead to point mutations of a base-substitutional kind. This has been shown by *in vitro* and *in vivo* studies on O^6-methylguanine (Loveless, 1969; Saffhill 1975), and similar studies on O^2-methylthymine and various O^4-alkylthymines (Singer *et al.*, 1986; Huh *et al.*, 1986; Dolan & Pegg, 1985). The template in these experiments served as a substrate for both RNA and DNA polymerases, but the demonstrated principle of aberrant base-pairing (or base-mismatching) remains the same, where the *O*-alkylated guanines base-pair like adenine (incorporating thymine instead of cytosine during pairing), and the *O*-alkylated thymines behave like cytosine (incorporating guanine instead of adenine).

The carcinogenic role of these *O*-alkylated bases is also corroborated by studies on the persistence of the *O*-alkylated residues in organs or tissues of rodents treated with alkylating NOC. Single-dose, multiple-dose and chronic exposure regimes have demonstrated the positive correlation between persistence of O^6-methylguanine and tumour incidence of rats treated with dimethylnitrosamine and nitrosomethylurea (Moiseev & Benemansky, 1976; Rose *et al.*, 1980; Irving *et al.*, 1979). Similar correlations between persistence and tumour incidence have also been noted for the case of O^4-methylthymine (Singer, 1986).

The above evidence, when taken together, serves to furnish a very convincing case for the role of modified DNA for NOC carcinogenesis. This role is believed to be mediated by the promutagenic properties of the products of DNA modification at the oxygen sites on DNA base residues. Figure II.1 depicts the likely sequence of molecular events underlying carcinogenesis by NOC in the light of the above quantum of experimental evidence.

IV.3: Genotoxical Relevant Factors and its Mechanistic Model.

From the Somatic Mutation Theory of carcinogenesis, it is possible to draw up a series of mechanistic factors involving DNA modification which would be relevant for the absence or presence of carcinogenic activity in the parent *N*-nitroso compounds. The formulation of these criteria is largely dependent upon the results of experiments pertaining to the pro-mutagenic character of O^6-alkylguanines and O^4-alkylthymines as described in section IV.1 above. The mechanistic factors relevants here are three in number as incorporated into the following model.

Mechanistic Factor One: Role of O-selectivity of DNA alkylation.

The mechanism of alkylation at the nucleophilic sites on DNA is most like an S_N2 one, with varying degrees of S_N1 character. The greater the degree of S_N1 character, the greater would be the degree of O-selectivity of the reaction, as predicted by a number of mechanistic and theoretical studies. Where it has been advanced that complexity and branching of the α-alkyl group in the ultimate carcinogen would promote S_N1 character and O-selectivity. The importance of O-selectivity of DNA modification follows the carcinogenic and mutagenic relevance of the oxygen site on DNA bases. Alkylating agents with greater S_N2 character would thus not be effective as carcinogens and mutagens, as it appears to be the case. This probably provides clues to the carcinogenicity of methyl-methanesulfonate, with its essentially complete S_N2 character as an alkylating agent and its very low O-selectivity of alkylation.

Mechanistic Factor Two Role of Watson-Crick proton acidity.

The role of Watson-Crick proton acidity for mutagenesis by alkylated DNA was first pointed out by the theoretical study of Duncan and Davies using semi-empirical molecular orbital theory. It may be argued that loss of Watson-Crick protons at the N^1-guanines and at the N^3-thymines would pave the way for potentially mis-matching situations for the modified bases involved. A clearcut demarcation can be arrived at between the non-mutagenic free unalkylated bases and N^7-alkylguanines and the pro-mutagenic O-alkylated bases, through theoretical calculated indices for Watson-Crick proton acidity. Thus the loss of Watson-

Crick protons emerges as a key mechanistic event for the adoption of promutagenicity by alkylated guanines and thymines.

Mechanistic Factor Three **Conformational role of O-alkyl group in O-alkylated bases:**

Theoretical modelling of the steric and energetic requirements for successful base-mismatching has predicted the important of conformation of the exocyclic O-alkyl group in O-alkylated bases. The work on *syn* conformer of O^6-methylguanine and O^4-methylthymine was predicted to have no base-pairing or mispairing properties whatsoever in the double-helical configuration, since interaction with all DNA bases was calculated to be repulsive. The *anti* conformers, however, were predicted to base-pair favourably with non-complementary bases as is in line with their experimentally attested pro-mutagenicity . Thus feasibility of the *syn* to *anti* rotation emerges as an important criterion to decide pro-mutagenicity.

IV.4 Role of O-selectivity DNA Alkylation:

The biological significance of alkylation at certain sites in DNA has been well characterised under experimental conditions, those include the oxygen atoms at position six of guanine position four of thymine and position two of uridine and cytosine (Singer, 1975; Kusmierek & Singer, 1976; Singer, 1976).

Loveless attributed the alkylation on the O^6 position of guanine to mutation of T_2 bacteriophage by *N*-nitroso-*N'*-methylurea and *N*-nitroso-*N'*-ethylurea (Loveless, 1969). The alkylation of O^4-thymine and O^2 Uracil sites following reaction

with diazomethane and diazoethane in non-aqueous solution has also been reported (Singer, 1976; Lawley, *et al.* 1973).

As pointed out in the previous chapter, that NOC react with DNA only after forming metabolically or spontaneous conversion to the alkanediazonium ion species, termed as the ultimate carcinogen. The mechanism of alkylation at the nucleophilic sites on DNA is most like an S_N2 one, with varying degrees of S_N1 character. The greater the degree of S_N1 character, the greater would be the *O*-selectivity of the reaction, as predicted by a number of mechanistic and theoretical studies, where it has been advanced that complexity and branching of the α-alkyl group in the ultimate carcinogen would promote S_N1 character and *O*-selectivity. The importance of *O*-selectivity of DNA modification follows from the carcinogenic and mutagenic relevance of the oxygen sites on DNA bases. Alkylating agents with greater S_N2 character would thus not be very effective as carcinogens and mutagens, as indeed appears to be the case. This probably provides clues to the noncarcinogenicity of methyl methanesulphonate, with its essentially complete S_N2 character as an alkylating agent and its very low *O*-selectivity of alkylation.

It is also known that alkylating agents with greater potential for mutagenicity and carcinogenicity produce greater O-selectivity of DNA alkylation than non-carcinogenic or less carcinogenic agents. This may be seen by comparing NOC to alkyl methanesulfonates.

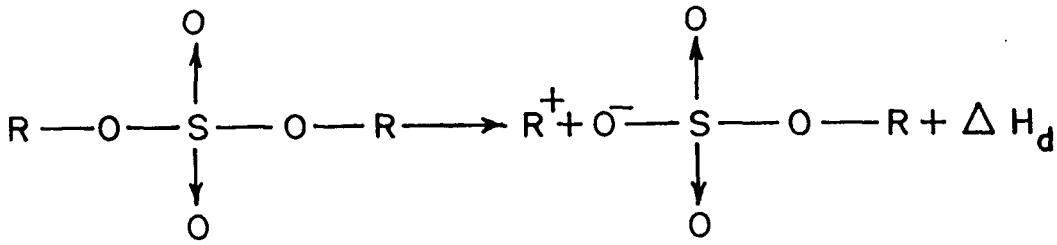
The ring *N*-atoms and the exocyclic *O*-atoms of the purines and pyrimidines are basicity in nature, as such they will also be

preferred sites for alkylating agents like *N*-nitroso compounds. Thus ethylating , *n*-propylating and *n*-butylating agents will have greater preferrence to react at O-atoms than the methylating agents as examplified by the reaction sites of O^6-alkylguanine and O^4-alkylthymine. Therefore, isopropylating and sec-butylating agents will have even greater preferrence to react with O-atoms, since more branched alkyl groups exhibit stronger active species as an acid which should have a greater preferrence for reaction at the O-sites (strong base).

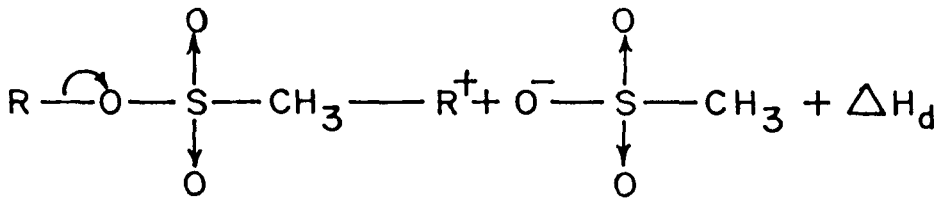
The reaction of DNA modification by the ultimate carcinogenic diazonium ions involves competition between alkylation at pro-mutagenic sites verses alkylation at non-mutagenic sites. For simple alkylating agents like methylating and ethylating *N*-nitroso compounds, the primary competitions is between non-mutagenic nitrogen sites like the N^7-guanine site and Oxygen sites like O^6-guanine and O^4-thymine sites. For methylating *N*-nitroso compounds, the N^7-guanine site is the chief sites for alkylating DNA (Benarek *et al.*, 1980; Morimoto *et al.*, 1983; Ortlieb & Khleihues, 1980), although a small proportion of O-alkylated products are also formed like O^6-methylguanine and O^4-methylthymine. For ethylating *N*-nitroso compound, the proportion of O-alkylated products is appreciably higher, including alkylation products at the phosphate ester moiety apart from O-alkylated DNA bases. With the increase in branching and complexity of the alkyl groups at the α-position (as exemplified by the isopropyl group) O-alkylated products are seen to be the chief products.

Equations IV·1 - IV·5 : DISSOCIATION REACTIONS OF :—

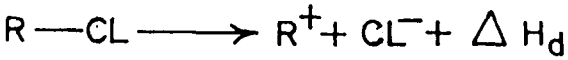
1. Dialkyl Sulfates



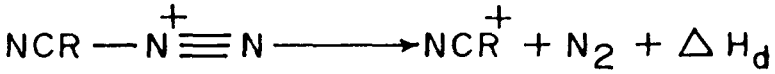
2. Alkylmethane Sulphonates



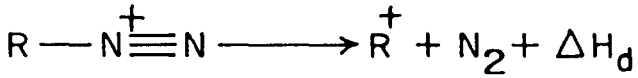
3. Alkyl chloride



4. Cyano–alkyldiagonium ion



5. Alkane diazonium ions



IV.5 Theoretical Calculations on Alkylation.

Theoretical calculations have been pursued here at the semi-empirical molecular orbital level using the AM_1 SCF-MO method, with full geometry optimisation by the Davidon-Fletcher-Powell algorithm. The aspects of the role of modified DNA, as dealt with above, are subjected here to investigation by these calculations, using appropriate physicochemical quantities of relevance as shown below :

Five kinds of alkylating agents are considered here to predict and compare their tendencies towards *O*-selectivity of alkylation. These include the direct-acting alkyl methanesulphonates, dialkylsulphates and alkylchloride, as well as the alkanediazonium ions and cyanomethyl diazonium ion. The tendency towards *O*-selectivity of alkylation has been linked to the S_N1 character of the alkylating agent and of the transition state involved in DNA alkylation. One factor which lends S_N1 character to an alkylating agent is the ease with which it would dissociate to yield the alkyl cation and the leaving group. This quantity may be represented by ΔH_d, the enthalpy of the dissociation reaction, as given in Figs.IV.2 (a) to (e) below for alkyl methanesulphonates, dialkyl sulphates and alkanediazonium ions respectively.

a. $R\text{-}SO_3Me = R^+ + (SO_3Me)^- + \Delta H_d$ (1)

b. $R\text{-}O\text{-}SO_2\text{-}O\text{-}R = R^+ + (RSO_4)^- + \Delta H_d$ (2)

c. $R\text{-}NN^+ = R^+ + N_2 + \Delta H_d$ (3)

d. $R\text{-}Cl = R^+ + Cl^- + \Delta H_d$ (4)

e. $CNR\text{-}CH_2NN^+ = R^+ + CNRCH_2^- + \Delta H_d$ (5)

Figure.IV.2(a) to (e). S^N1 dissociation reactions.

Table.IV.3: AM1 calculated values for effects of O-selectivity parameters upon carcinogenic activity of alkylating agents.

ALKYLATING AGENTS	ALKYL GROUPS	FUNCTIONS ΔH_d	Q_c	Q_r	W_{cx}
1. Alkanediazo	Me	39.410	-0.0966	0.2003	0.8987
nium ions	Et	14.120	-0.0138	0.1881	0.8867
	n-Pr	12.665	0.2786	0.1876	0.8982
	n-Bu	-5.529	0.3051	0.1889	0.8920
	n-Pe	-17.242	0.2773	0.1889	0.9098
	Pr^i	-3.229	0.0611	0.1588	0.9049
	Bu^t	-16.069	0.1332	0.1565	0.9157
2. Cyano-methyl	Me	16.2389	0.2313	0.2130	0.9195
diazonium	Et	15.6311	-0.2569	0.1737	0.9073
ions	n-Pr	14.2269	-0.0210	0.1880	0.9087
	n-Bu	14.2403	-0.0199	0.1884	0.9173
	n-Pe	13.1117	-0.0195	0.1888	0.9065
	Pr^i	-2.2661	0.4547	0.1989	0.9045
3. Alkyl	Me	233.644	-0.2699	0.1438	0.9315
chloride	Et	205.399	-0.1134	0.1020	0.9666
	n-Pr	201.804	-0.1132	0.1035	0.9683
	n-Bu	200.807	-0.1124	0.1041	0.9680
	n-Pe	200.517	-0.1121	0.1942	0.9677
	Pr^i	185.557	-0.2219	0.1078	0.9492
	Bu^t	139.759	-0.2170	0.1069	0.9560

4. Alkyl	Me	219.781	-0.0986	0.1492	0.9583
methane	Et	189.301	-0.0458	0.1494	0.9583
sulfonates	n-Pr	187.207	-0.0476	0.1493	0.9583
	n-Bu	186.148	-0.0472	0.1494	0.9583
	n-Pe	185.830	-0.0474	0.1494	0.9582
	Pr^i	168.310	0.0178	0.1489	0.9600
	Bu^t	152.296	0.1261	0.1422	0.9670
5. Dialkyl	Me	545.213	-0.1223	0.1157	1.0246
sulfates	Et	483.321	-0.0584	0.1083	0.9745
	n-Pr	480.065	-0.0528	0.1059	0.9795
	n-Bu	477.989	-0.0516	0.1037	0.9797
	n-Pe	477.414	-0.0525	0.1064	0.9796
	Pr^i	444.712	-0.0053	0.1387	0.9764
	Bu^t	408.821	0.0698	0.1053	0.9511

Further physicochemical quantities giving clues to facility of dissociation and S_N1 character are the Wiberg bond strength index W_{CX} for the bond broken during dissociation, and the Mulliken charges Q_α and Q_r on the α-carbon atom and on the whole alkyl group respectively. The AM_1 values of the W_{CX}, Q_α, Q_r and H_d indices for S_N1 character are presented in Table IV.3(a) & IV.3(b) for the five classes of alkylating agents studied, each type being represented by the methyl (Me), ethyl (Et), isopropyl (Pr^i), *ter*-butyl (Bu^t), *n*-propyl (Pr), *n*-butyl (Bu) and n-pentyl (Pe) homologues.

It is noteworthy that the agents predicted to have the lowest S_N1 character is methyl methanesulphonate, whose carcinogenic and mutagenic activities have not been detected. The predicted low level for S_N1 character and consequent *O*-selectivity of alkylation correlates well with the genotoxic inactivity of this agent as experimentally established (Pegg, 1975; Lawley, 1974 & 1976). These predictions are thus well in accord with the postulated role of the *O*-alkylated bases for mutagenicity and carcinogenicity (Osterman-Goldkar *et al.*, 1970; de Vos *et al.*, 1988).

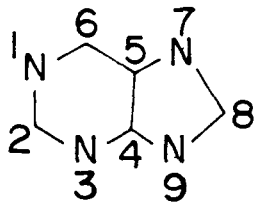
For any one alkyl group, the order predicted for S_N1 character is alkanediazonium ion > cyanomethyldiazonium ion> alkylchloride> dialkylsulfate > methanesulphonate, which order may thus be expected for the trend in *O*-selectivity of alkylation. For all three classes of agents, the order predicted for S_N1 character and *O*-selectivity of alkylation is Bu^t > Pr^i > Et > Me. Increase in length and, particularly, branching of the alkyl

group at the α-carbon are thus predicted to be concomitant with greater S_N1 character and *O*-selectivity of the alkylating agent. The *ter*-alkylating agents are predicted to display maximum *O*-selectivity of alkylation. This appears to go far from explaining why any NOC with a *ter*-alkyl group structure at the α-carbon is inactive as a carcinogen. For this, appeal has to be made to the second and third criteria mentioned above, *viz.* the role of Watson-Crick proton acidity, and the role played by the barrier to *syn-anti* rotation of the *O*-alkyl group in the *O*-alkylated bases.

IV.6: Role of Watson-Crick Proton Acidity :

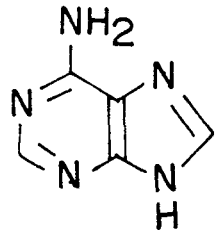
The role of Watson-Crick proton acidity for mutagenesis by alkylated DNA was first pointed out by the theoretical study of Duncan and Davies using semi-empirical molecular orbital theory (1989). It was further argued by Lyngdoh (1992) and by Lyngdoh Venkateshwarlu (1994) that loss of the Watson-Crick proton (the N^1-G proton of guanines and the N^3-T proton for thymines) would pave the way for potentially mis-matching situations in the modified bases involved. Using theoretically calculated indices for Watson-Crick proton acidity, a clear cut demarcation was arrived at between the non-mutagenic free unalkylated bases N^7-alkylguanines (Ludlum, 1970; Reddy *et al.*, 1982; Druckrey *et al.*, 1967) that do not lead to miscoding of bases on one hand and the pro-mutagenic (Boyland *et al.*, 1968; Dontenwill, 1968) O-alkylated bases O^6-alkylguanines (Mehta & Ludlum, 1976 & 1978; Abbott & Saffhill, 1979) and O^4-alkylthymines (Davson *et al.*, 1963; Reddy *et al.*, 1982; Okada & Ishidate, 1977) leading to

Parent structure and numbering system



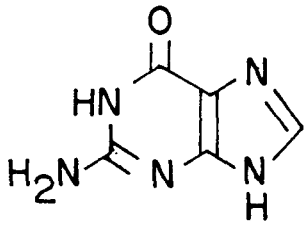
Purines

DNA base

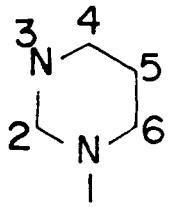


Adenine (A)

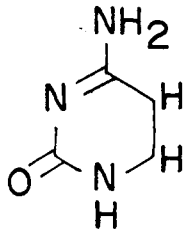
DNA base



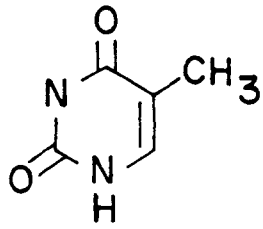
Guanine (G)



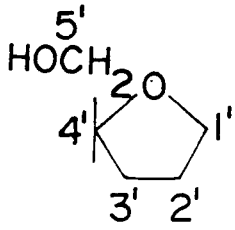
Pyrimidine

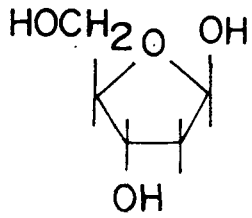


Cytosine (C)



Thymine (T)





B – 2'– deoxyribose
(in DNA)

Fig IV-3: Parent structure and numbering system of DNA bases, with abbreviations.

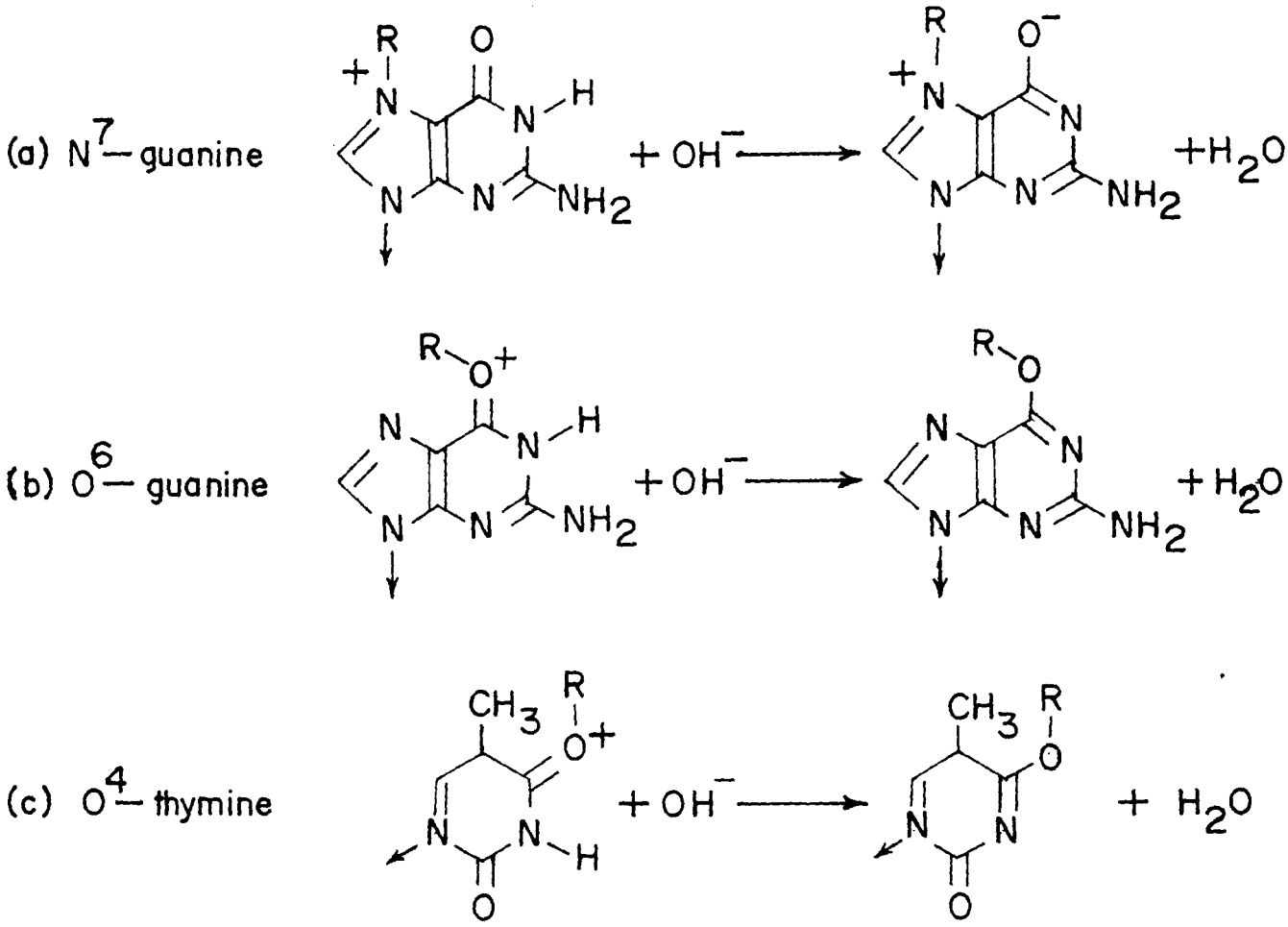
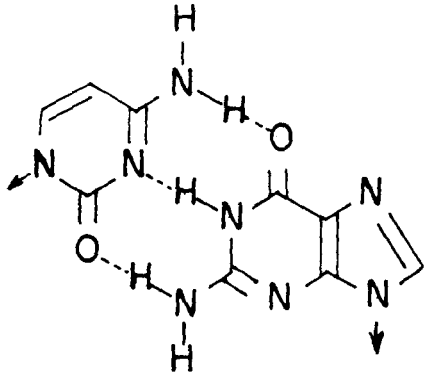
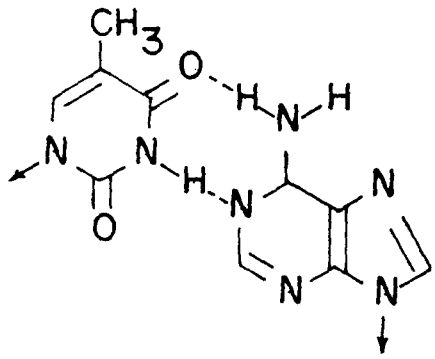


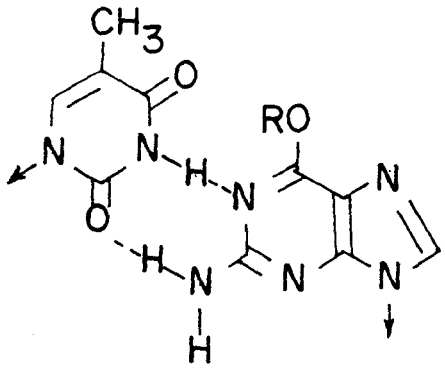
Fig IV-4: The Watson – Crick deprotonation reaction



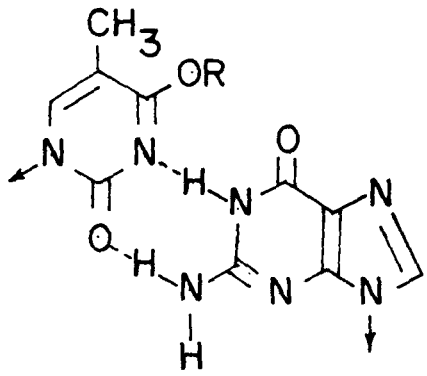
(a) Cytosine : Guanine



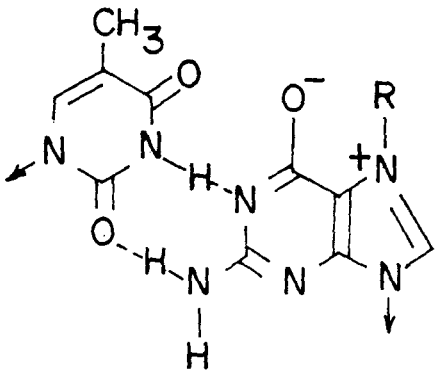
(b) Thymine : Adenine



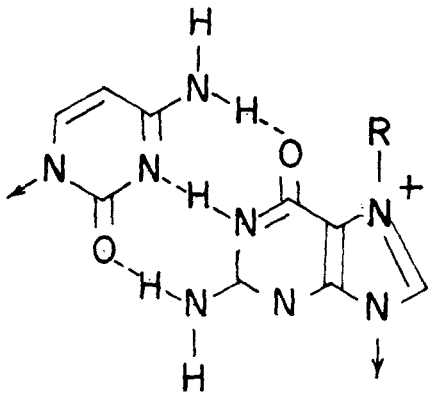
(c) Thymine : O^6–Alkylguanine



(d) O^4–Alkylthymine : Guanine



(e) Thymine : Neutral
N^7– alkylguanine



(f) Cytosine : Cationic
N^7– alkylguanine

Fig IV.5: Normal and abberant base-pairing schemes for free and alkylated DNA bases.

wrong nucleotides incorporation during DNA synthesis on the other. Loss of the Watson-Crick protons thus emerges as a key mechanistic event for the adoption of pro-mutagenicity by alkylated guanines and thymines.

Apart from experimental work on the base-pairing properties of modified DNA bases, theoretical studies have also been pursued on this. Figure IV.4, presents the parent structure and numbering system of the DNA bases, while figure IV.5, is a representation of the Watson and Crick deprotonation reaction. The notable demarcation between promutagenic and non-mutagenic DNA base sites may thus been attributed to loss of the Watson-Crick protons, at biological *p*H as contrasted with retention of Watson and Crick protons at biological *p*H for the non-mutagenic species. This loss of the Watson-Crick Protons at biological *p*H for O^6- alkyl-guanines and O^4-alkylthymines is belived to be responsible for their promutagenicity. Loss of the N^1-guanine proton from O^6-alkylthymines makes the normal base pairing schemes unfavourable.

At the same time, it facilitiates abberrant or unusual base-pairing schemes where the modified guamine base-pairs like adenine (incorporating thymine instead of cytosine), and the modified thymine base-pairs like cytosine (incorporating guanine instead of adenine). However N^7-alkylguanine at biological *p* does not undergo N^1-G proton loss and therefore behaves like guanine. This normal and abberant base-pairing schemes for free and alkylated DNA bases are portrayed in figure IV.6.

IV.7: Theoretical Calculations on Mutagenic Role of Watson and Crick Proton Abstraction.

Comparison of Watson-Crick proton acidity is made here for three kinds of alkylated DNA bases - the promutagenic O^6-alkylguanines and O^4-alkylthymines and the nonmutagenic N^7-alkylguanines. The possible effects of alkyl group structure are studied by incorporating the methyl, ethyl, isopropyl and *ter*-butyl homologues for each type of modified base. The Watson-Crick protons are the N^1-G proton for the alkylated guanines and the N^3-T proton for the alkylated thymines.

Watson-Crick proton acidity is compared by calculating the enthalpy ΔH_{dp} of abstraction of the concerned proton by a hydroxide anion, as given by Eqn. (4) below :

$$(RBH)^+ + OH^- \quad RB + H_2O + \Delta H_{dp} \qquad (4)$$

where $(RBH)^+$ and RB stand for the undeprotonated and deprotonated forms of the alkylated base respectively. Further indices for tendency of proton loss are given by the Mulliken charge Q_h on the proton, the Wiberg bond index W_{xh} for the bond broken during deprotonation, and the frontier orbital term T_{fo} for non-bonded overlap interaction between the proton and the hydroxide anion, as given in Eqn. (5) below :

$$T_{fo} = (C_o C_h)^2/(E_o - E_h) \qquad (5)$$

Table IV.7 furnishes the AM_1 calculated values on the effects of the Watson-Crick proton acidity upon promutagenic properties of alkylated bases for N^7-RG, O^6-RG and O^4-RT. The indices are

Table IV.7: AM$_1$ calculated values on the effects of Watson-Crick protons acidity upon pro-mutagenic properties of alkylated bases for N^7-RG, O^6-RG and O^4-RT.

Compound	Functions	Homologues			
		Me	Et	Pr^i	Bu^t
1. N^7-alkyl	W_{ch}	0.8996	0.8996	0.8997	0.8997
guanine	Q_h	0.2299	0.2297	0.2296	0.2295
	ΔH_{dp}	238.69	239.57	240.00	240.17
2. O^6-alkyl	W_{ch}	0.8990	0.8991	0.8997	0.8954
guanine	Q_h	0.2336	0.2335	0.2332	0.2409
	ΔH_{dp}	203.63	204.69	205.55	208.68
3. O^4-alkyl	W_{ch}	0.8920	0.8921	0.8922	0.8923
thymine	Q_h	0.2483	0.2480	0.2477	0.2465
	ΔH_{dp}	204.92	206.11	207.26	208.28

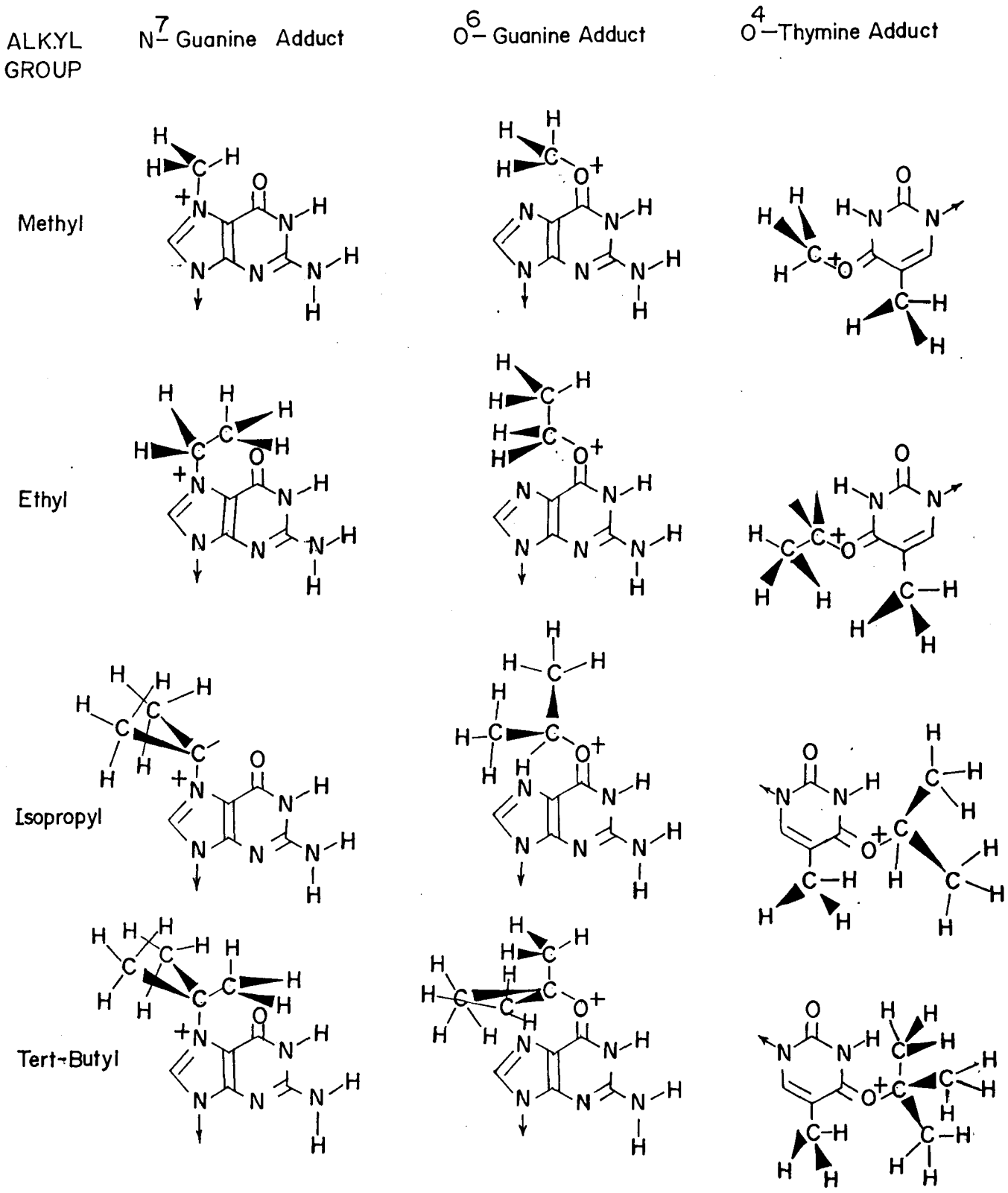
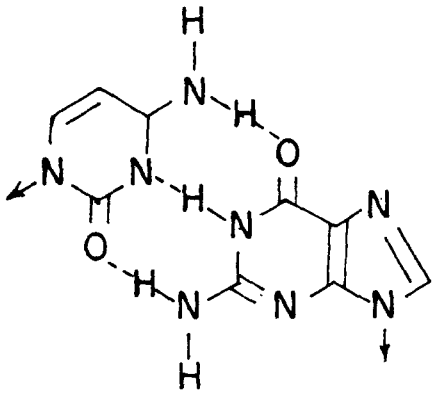
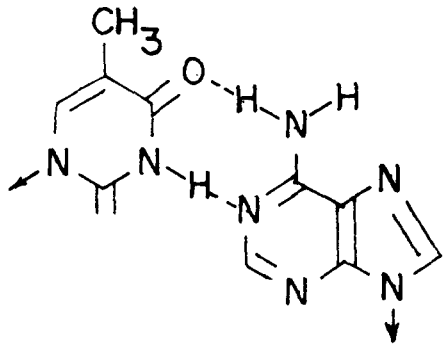


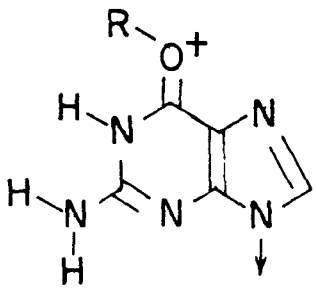
Fig IV.7: Stable conformers of DNA bases (adducts) alkylated N^7–G, O^6–G and O^4–T sites.



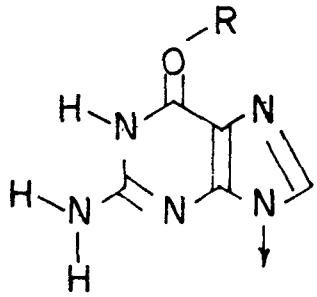
(a) Cytosine : Guanine



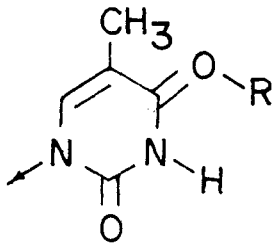
(b) Thymine : Adenine



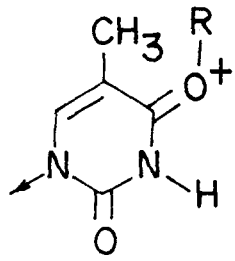
(c) Syn O^6-alkylguanine



(d) Anti O^6-alkylguanine



(e) Syn O^4-alkylthymine



(f) Anti O^4-alkylthymine

Fig IV.8c : The Syn and anti stable conformers for O-alkylated bases.

noted as ΔH_{dp}, Q_h, W_{xh} and T_{fo} for Watson-Crick proton acidity, values being presented for the methyl, ethyl, isopropyl and *ter*-butyl homo-logues of the N^7-alkylguanines, O^6-alkylguanines and O^4-alkyl-thymines, while Figure IV.8. represents the stable conformers of DNA bases (adducts) alkylated at N^7-RG, O^6-RG and O^4-RT sites. The first point to note is the straight forward demarc-ation in index values between the nonmutagenic N^7-alkylguanines and the promutagenic *O*-alkylated bases. The former are predicted on all counts to have lower Watson-Crick proton acidity than the latter, the actual predicted order being O^4-RT > O^6-RG > N^7-RG. This trend is well in line with the genotoxic properties of the three classes of alkylated bases, and serves to corroborate the proposal that Watson-Crick proton loss is in fact a key molecular event which confers mutagenic properties on to a modified DNA base, as was first proposed by Duncan and Davies.

The further point to note is that the *ter*-butyl homologues of the *O*-alkylated bases do not differ much from the other homolo-gues in that they would also be expected to exist in deprotonated form at neutral *p*H. The carcinogenic and mutagenic inactivity of *ter*-alkylating NOC is thus not explicable from the point of view of Watson-Crick deprotonation facility of the *O*-alkylated bases. Since the criteria one and two mentioned above fail to explain this inactivity, recourse has to be made now to criterion *three.*

IV.8 Mutagenic Role of O-alkyl Group Conformation in O-alkylated Bases:

The promutagenic role of the rotational barrier of DNA bases around the glycosyl bond was first postulated by Topal and Fresco (1976) to explain spontaneous tranversions in which the

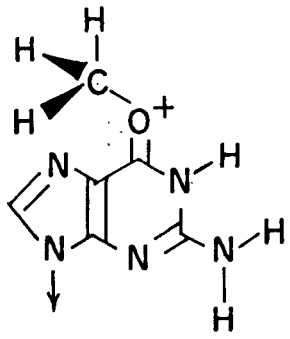
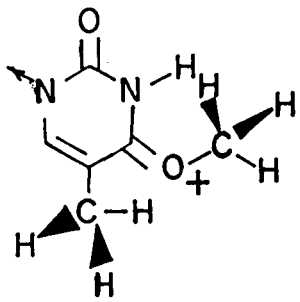
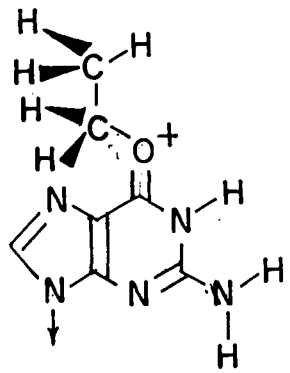
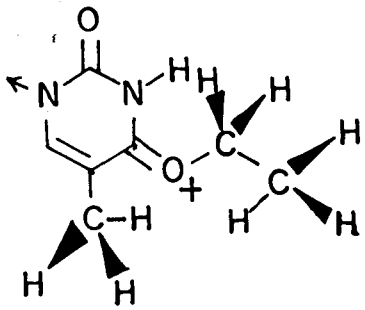
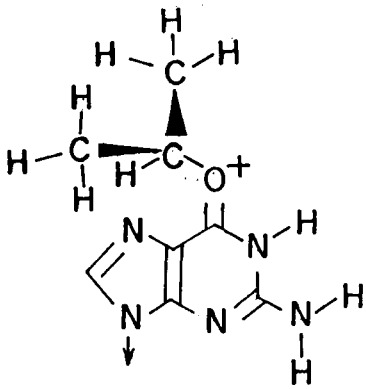
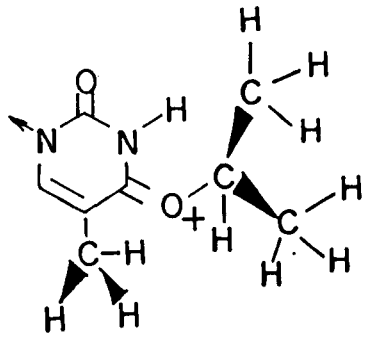
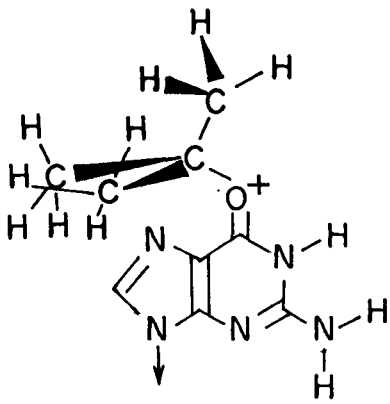
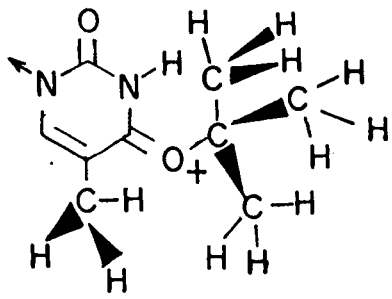
ALKYL GROUP	O^6-GUAMINE ADDUCTS	O^4-THYMINE ADDUCTS
Methyl		
Ethyl		
Isopropyl		
Tert-Butyl		

Fig-IV-9: : Stable conformers of DNA bases (adducts) alkylated at O^6-G and O^4-T sites.

nucleoside can exist either in rare tautomeric form *anti* conformation or in the *syn* conformation. The belief was that the double stranded left-handed DNA helix exist in syn and the more common right-handed DNA helix in the anti- orientation in their alternative rotamers *d(G,C)* (Grunberger & Santella, 1981; Behe & Felsenfeld, 1981). Chemical modification of nucleotides could be a likely factor to promote the formation of a partial segment of left-handed helix, which is a consequence of the rotation of the base moiety around the glycosyl bond (Zacharias *et al.*, 1982).

IV.9 Theoretical Calculations syn-anti- *Rotations of O-alkylated Bases.*

An attempt is now made to invoke the conformational role of the exocyclic *O*-alkyl group of *O*-alkylated bases to explain the genotoxic inactivity of *ter*-alkylating *N*-nitroso compounds. Using the AM_1 SCF-MO method with full optimisation, the barriers to *syn-anti* rotation are estimated for a set of O^6-alkylguanines and O^4-alkylthymines, where the methyl, ethyl, isopropyl and *ter*-butyl homologues are subjected to study. For each angle of rotation, full geometry optimisation was carried out except for the dihedral angle defining the angle of rotation. Values are tabulated for the heats of formation corresponding to the *syn* rotamer $-\Delta H_f(syn)$, to the maximum point on the rotational barrier - $H_f(max)$, and to the *anti* rotamer - $\Delta H_f(ant)$. Values are also given for the energy barrier to the *syn-anti* rotation - $E_r(s\text{-}a)$, the barrier to the *anti-syn* rotation - $E_r(a\text{-}s)$, and the enthalpy difference between the *syn* and *anti* rotamers - $\Delta H(a\text{-}s)$. All these values are presented in table IV.11 & IV.12. The rotations are represented in Figs.IV.9. for *syn- to anti-* stable conformers

Table.IV.11: AM_1 calculated data of *Syn-anti*rotational barrier for O^6-alkylguanines.

Molecule	ΔH_f(*syn*)	ΔH_f(*anti*)	$\Delta H_{f(max)}$	θ_{max}	$E_{s->a}$	$E_{a->s}$	$\Delta\Delta H_{f(sa)}$
O^6-MeG	58.20	61.50	62.65	97	4.45	1.15	3.30
O^6-EtG	52.93	57.00	57.55	95	4.62	0.55	4.07
O^6-Pr^iG	49.66	57.84	57.84	180	8.18	0.00	8.18
O^6-Bu^tG	48.39	57.54	57.54	180	9.15	0.00	9.15

Table.IV.12: AM_1 calculated data of *Syn-anti* rotational barrier for O^4-alkylthymine

Molecule	$\Delta H_{f(syn)}$	$\Delta H_{f(anti)}$	$\Delta H_{f(max)}$	θ_{max}	$E_{s->a}$	$E_{a->s}$	$\Delta\Delta H_{f(sa)}$
O4-MeT	42.63	31.48	31.48	180	11.15	0.00	11.15
O4-EtT	48.38	35.83	35.83	180	12.55	0.00	12.55
O4-PriT	52.19	39.47	39.47	180	12.72	0.00	12.72
O4-ButT	52.66	34.67	34.67	180	17.99	0.00	17.99

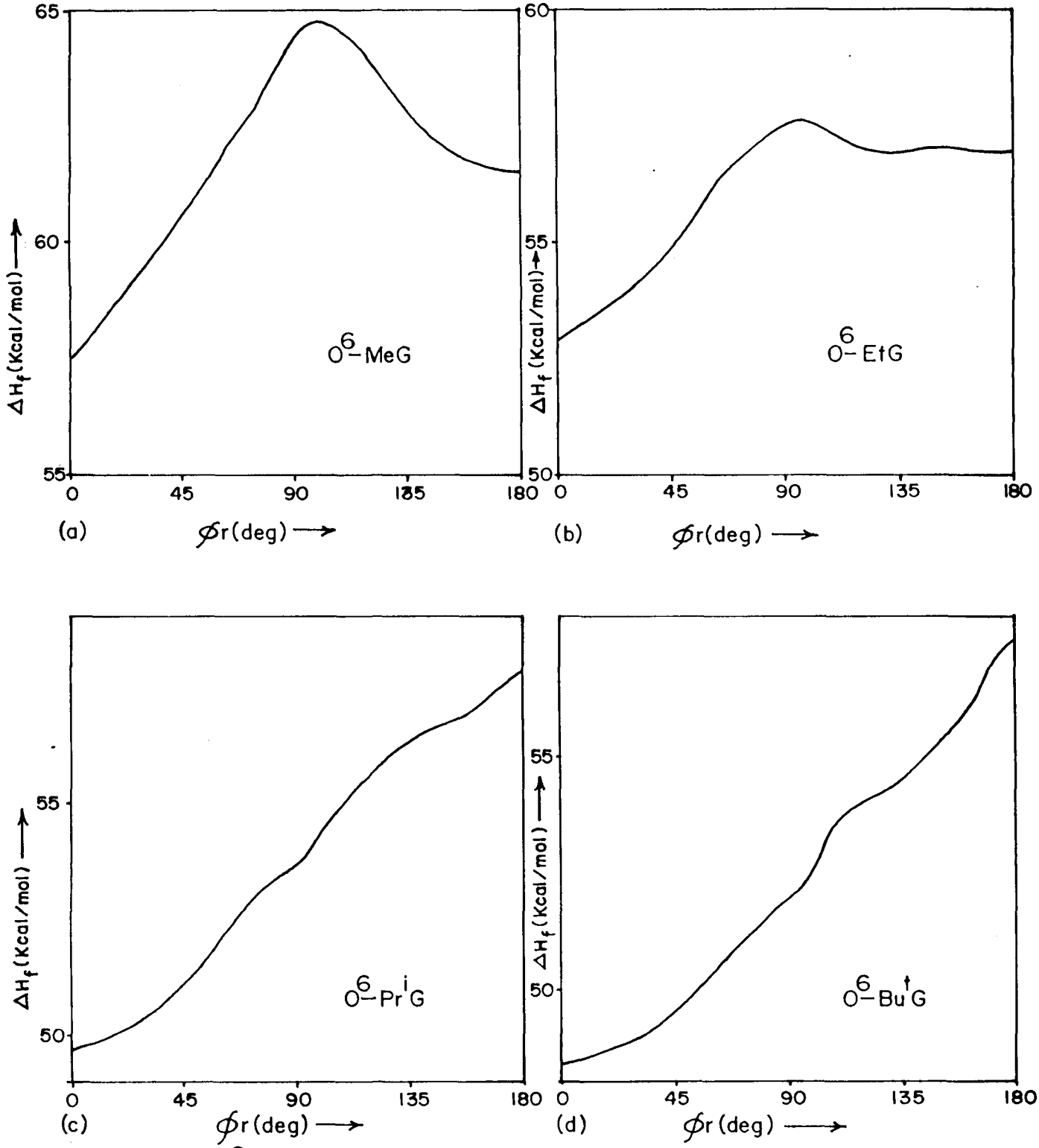


Fig-IV.12: Plot of O^6—alkylguanine Dihedral angle [ϕr (deg)] verses final heat of formation [ΔH_f (Kcal/mol)] (a) O^6—MeG (b) O^6—EtG (e) O^6—Pr^iG and (d) O^6—Bu^tG.

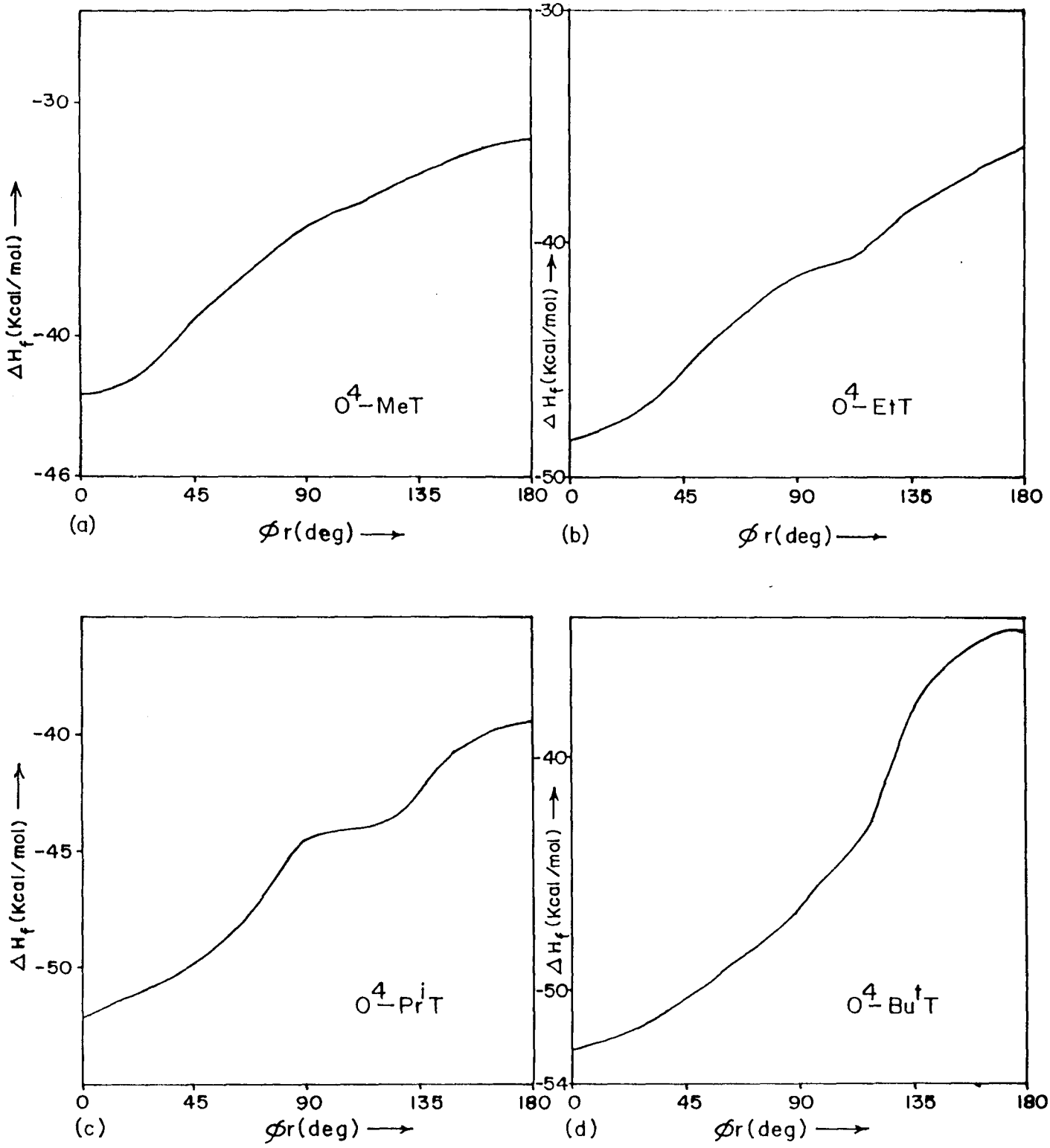


Fig IV.13 Plot of O^4-alkylthymine, Dihedral angle [Ør(deg)] verses final heat of formation [ΔH_f (Kcal/mol)] (a) O^4-MeT (b) O^4-EtT (c) O^4-Pr^{i}T and (d) O^4-Bu^{t}T .

for O-alkylated, and Figs. IV.10. for the O^6-alkylguanines, and for the O^4-alkylthymines, where the heat of formation of the system ΔH_f is plotted against the dihedral angle ϕ_r for the rotation of the *O*-alkyl group about the *C-O* bond, taken as 0^o deg. for the *syn* rotamer given in Figs.IV.13 for O^6-alkylguanines and Figs.IV.14 for O^4-alkylthymines

The first point to note is that the *anti* conformers are invariably less stable than the *syn* ones. This presupposes the overcoming of some energy barrier for all cases to pass from the non-mispairing *syn* conformer to the mispairing *anti* one. Furthermore, the *syn* to *anti* rotational barriers are lower for the O^6-alkylguanines than for the O^4-alkylthymines, which is attributable to the presence of the C^5-methyl group in the latter affording some degree of steric hindrance.

The bases O^6-MeG and O^6-EtG are similar in that they display two minima during rotation - one flat and higher minimum around and the region corresponding to the *anti* rotamer and another sharper and lower minimum corresponding to the *syn* rotamer. The *syn-anti* rotation barriers are 2.5 and 3.5 kcal/mol for the methyl and ethyl cases, while the *anti-syn* barriers are 7.2 and 8.3 kcal/mol respectively for the methyl and ethyl bases. These values correspond well with the MNDO and INDO values reported for O^6-MeG by Pohorille and Loew and by Duncan respectively. The low predicted value of the *syn-anti* rotational barrier would allow for a substantial degree of the *syn-anti* rotation taking place at room or biological temperature, especially so when assisted by the energy lowering afforded while base-mispairing occurs.

All the other systems show only one minimum, corresponding to the *syn* conformer. The value of the *syn* to *anti* rotation is consistently higher for the O^4-alkylthymines than for the O^6-alkylguanines. The values for the O^6-alkylguanines give the order Bu^t > Pr^i > Et > Me, which order is also followed by the O^4-alkyl thymines, although on a somewhat higher scale of values. These calculated values predict that of all the four types of alkyl groups, the *ter*-butyl group would experience the most difficulty in assuming the base-mispairing *anti* conformer for both O^6-alkylguanines and O^4-alkylthymines. The value for O^4-Bu^tT is particularly high, and suggests the impossibility of it occurring at all in the real situation.

The *syn* conformer of O^6-methylguanine and O^4-methylthymine was predicted to have no base-pairing or mispairing properties whatsoever in the double-helical configuration, since interaction with all DNA bases was calcula-ted to be repulsive. The *anti* conformers, however, were predicted to base-pair favourably with noncomplementary bases as is in line with their experimentally attested promutagenicity. Feasibility of the *syn* to *anti* rotation thus emerges as an important criterion to decide promutagenicity. The *syn* rotamer is invariably the stabler one, and this is the one that may be assumed to be adopted during DNA unwinding prior to replication. As the DNA synthesis proceeds, the *O*-alkyl group would have to move out of the hydrogen-bonding zone if pairing with any base is to be effected. Uncomfortably high barriers to the *syn* to *anti* rotation would effectively preclude any base-pairing whatsoever, and act as a deterrent to successful base-mismatching.

Taking the cue from theoretical calculations, it may now be possible to apply this mechanistic criteria to a series of inactive NOC, all containing *tert*-alkyl groups. These include the *N*-nitroso-*N*-*n*-butyl-*tert*-butylamine (Okada *et al.*, 1977), *N*-nitroso-*N*-methyl-*tert*-butylamine (Gold *et al.*, 1981), *N*-nitroso-*N*-acetoxymethyl-*tert*- butylamine (Gold *et al.*, 1981), *N*-nitroso-*N*-ethyl-*tert*-butylamine (Druckrey *et al.*, 1967), and *N*-nitroso-2,2,6,6-tetramethylpiperidine (Lijinsky *et al.*, 1975), which are all found non-carcinogenic in tests animals.

The notably high values of the *syn-anti* rotational barrier for the case of the *ter*-butylated bases, especially the thymine case, would seem to furnish a good rationale for the inability of any NOC containing *ter*-alkyl groups to induce gene mutations or cancer in animals. The key mechanistic basis for this is the role played by the conformation of the *O*-alkyl group in the alkyl base.

IV.10 *Result and Discussion:*

The mechanistic role of the modified DNA establishes three main mechanistic criterions for conferring the mutagenic and carcinogenic properties on to DNA. The first key criteria is the sufficient *O*-selectivity of DNA alkylation for which verification a sample of calculations on *O*-selectivity trend for DNA alkylations towards SN_1 character identification was computed for five kinds of alkylating agents. The AM_1 SCF-MO calculated values of SN_1 character for these five kinds of alkylating agents resulted appreciably close in line with the experimental values, which correlates well with the genotoxic activity or inactivity of these agents as experimentally established.

The order predicted for SN_1 character is alkyldiazonium ion > cyanoalkyldiazonium ion > alkylchloride ion > dialkylsulfate > methanesulfonate and the order predicted for SN_1 character and *O*-selectivity of alkylations is Bu^t > Pr^i > *n*-Pen > *n*-Pr > Et > Me. It is thus concluded that the more the length and the branching of the alkyl groups at the α-carbon, the more is the SN_1 character and greater will be their *O*-selectivity.

The second emerging criteria is the role of Watson-Crick protons acidity for DNA modifications towards the pro-mutagenic and pro-carcinogenic effects. The three kinds of alkylated DNA bases studied for comparison here, are the pro-mutagenic O^6-alkylguanines and O^4-alkylthymines and the non-mutagenic N^7-alkylguanines. Methyl, Ethyl, Isopropyl, and *tert*-butyl homologues were incorporated to study the possible effects of alkyl groups structure in the event of DNA modification. Our AM_1 SCF-MO calculated results predict the order of Watson-Crick protons acidity as O^4-RT > O^6-RG > N^7-RG, which order coincide well with the genotoxic properties of these three alkylated bases. This calculated values also clearly demarcate the index values between the non-mutagenic N^7-alkylguanines and the pro-mutagenic O-alkylated bases. Thus it was concluded that the loss of Watson-Crick protons is the key molecular event that confers the mutagenic properties on to a modified DNA bases.

While in the third criteria, the AM_1 SCF-MO calculation output on the conformational role of O-alkyl group in O-alkylated bases has been very successful and satisfactory. The rotational barriers of *syn- to anti-* rotation are estimated for O^6-

alkylguanines and O^4-alkylthymines, using AM_1 SCF-MO method with full geometry optimisation for each angle of rotation. Methyl, Ethyl, Isopropyl, and *tert*-butyl homologues are subjected to study. The order of *syn- to anti-* rotational barriers were found to be O^4-alkylthymines > O^6-alkylguanines, and that of the homologues are Bu^t > Pr^i > Et > Me, in both the cases. The extremely high value of *tert*-butyl, particularly that of O*4-tert*-butylthymines is a clear case of impossible to exist in real situation, which strongly suggest that carcinogens containing *tert*-alkyl groups would not be able to modify DNA to induce gene mutation, thereby, unable to induce cancers in animals.

CHAPTER FIVE

A MECHANISTIC INDEX FOR ASSESSING RELATIVE CARCINOGENIC POTENCY OF N-NITROSO CARCINOGENS

Contents.

V.1. Relative Carcinogenic Potency of N-nitroso Compounds.

As a class, *N*-nitroso compounds are found to be largely carcinogenic in representative oncogenesis tests using animals, chiefly rodents (Bartsch *et al.*, 1984; Druckrey *et al.*, 1967). From the *dose-response* data obtained thus, attempts have been made to gauge relative carcinogenic potency among active members of the class. One of the most internally consistent dose-response studies is that of Druckrey *et al.*, 1963; 1973) on 65 different NOC performed on BD strain rats. Wishnok and Archer (1967) utilised this data to frame a numerical index I_c for relative carcinogenic potency given by:

$$I_c = \log(1/D_{50}) \qquad (1)$$

where D_{50} is the median carcinogenic dose in mmol/kg body weight as obtained by Druckrey *et al.*, (1967).

Table.V.1: Potency data of 13 dialkylnitrosamines specific for hepatocarcinogenicity.

NAME OF COMPOUNDS	ABBREVIATIONS	D_{50} (Mol/Kg body Wt.)	I_c
1. Dimethylnitrosamine	(DMeNA)	0.0054	2.268
2. Cyanomethylnitrosamine	(MeCyNA)	0.0066	2.180
3. Dicyanonitrosamine	(DCyNA)	0.0121	1.917
4. Diethylnitrosamine	(DEtNA)	0.00063	3.201
5. Dipropylnitrosamine	(DPrNA)	0.0088	2.056
6. Dibutylnitrosamine	(DBuNA)	0.02465	1.608
7. Dipentylnitrosamine	(DPeNA)	0.2576	0.589
8. Butyl-n-Pentyl-nitrosamine	(BuPeNA)	0.0987	1.006
9. Diisopropylnitrosamine	($DPr^{i}NA$)	0.1075	0.969
10. N-Nitrosobis-(2-hydroxyethyl)amine	(DHeNA)	1.1186	-0.048
11. N-Nitrobis-(2-acetoxyethyl)amine	(DAeNA)	0.1834	0.737
12. N-Nitroso-N-ethyl (2-hydroxyethyl)amine	(EtHeNA)	0.0156	1.807
13. Methylethylnitrosamine	(MeEtNA)	0.0048	2.319

Table.V.2: Potency data of 7 dialkylnitrosamines specific for Oesophagal cancer.

NAME OF COMPOUNDS	ABBREVIATIONS	D_{50} (Mol/Kg body Wt.)	I_c
1. Methylpentyl-nitrosamine	(MePeNA)	0.0025	2.602
2. Methylvinyl-nitrosamine	(MeViNA)	0.0023	2.638
3. Methylphenyl-nitrosamine	(MePhNA)	0.0250	1.602
4. Ethyl-n-butyl-nitrosamine	(EtBuNA)	0.0077	2.114
5. Ethylvinyl-nitrosamine	(EtViNA)	0.0023	2.638
6. Ethylisopropyl-nitrosamine	(EtPr^{i}NA)	0.0318	1.498
7. Methylallyl-nitrosamine	(MeAlNA)	0.0080	2.097

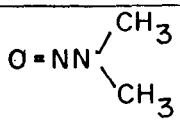
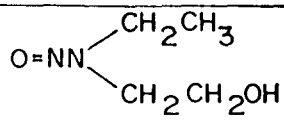
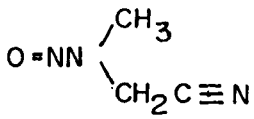
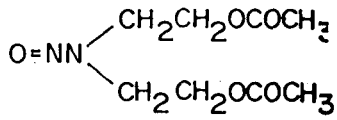
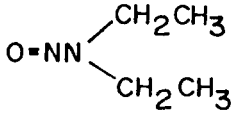
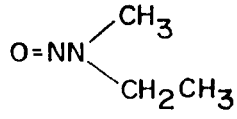
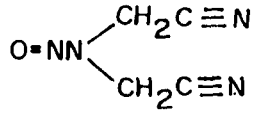
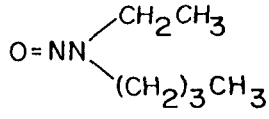
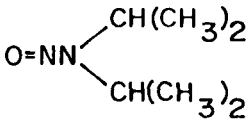
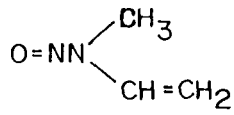
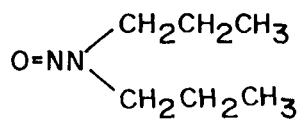
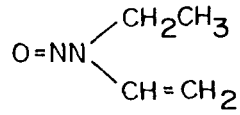
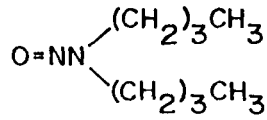
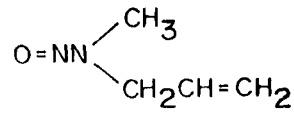
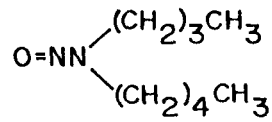
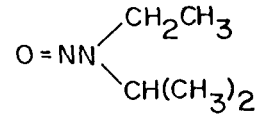
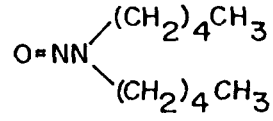
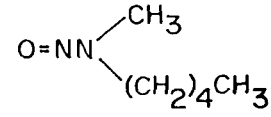
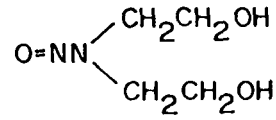
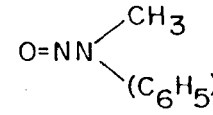
NO. Compound Name	Structure	NO. Compound Name	Structure
1. N-Nitrosodimethylamine		11. N-Nitroso-N-ethyl (2-hydroxy ethyl)amine	
2. N-Nitrosolyano-methylamine		12. N-Nitrosobis (2-acetoxyethyl)amine	
3. N-Nitroso-diethylamine		13. N-Nitroso-N-methylethylamine	
4. N-Nitroso-dicyanoamine		14. N-Nitrosoethyl-n-butylamine	
5. N-Nitroso-diisopropylamine		15. N-Nitroso-N-methylvinylamine	
6. N-Nitroso-di-propylamine		16. N-Nitroso ethyl vinylamine	
7. N-Nitroso-di-butylamine		17. N-Nitroso-N-methylallylamine	
8. N-Nitroso-n-butyl-n-pentylamine		18. N-Nitroso ethyl isopropylamine	
9. N-Nitroso-di-n-pentylamine		19. N-Nitroso-N-methyl-n-pentylamine	
10. N-Nitrosobis (2-hydroxyethyl)amine		20. N-Nitroso-N-methyl phenylamine	

Fig V.3: Structures of Dialkylnitrosamines studied. Structures 1—13 are Hepatocarcinogenic DRNA and Structures 14—20 are Oesophagalcarcinogenic DRNA.

Table V.1, presents potency data for 13 dialkylnitrosamines which are chiefly hepatocarcinogenic in rats, giving their full names, abbreviated names, median carcinogenic dose and values of I_C. Table V.2, presents similar data for 10 dialkylnitrosamines which are chiefly oesophagal carcinogens in rats and Figs.V.3 represent the chemical structures of 20 *N*-nitroso compounds studied above. The wide range of carcinogenic potency can be seen from the more than a thousand-fold difference between DEtNA (the most potent NOC) and DHeNA (the weakest member). Hepatic carcinogens are treated separately from oesophagal carcinogens in order to treat the phenomena separately on an equable basis for the various members of each sub-class. This is because, as will be seen later, the target organ for tumourigenesis is an important factor determining the magnitude of the carcinogenic effect. Since this factor cannot be quantified appropriately, it appears best here to isolate it out by this grouping into two separate sub-classes.

V.2. Mechanistic Determinants for Relative Carcinogenic Potency. Conceptual approach

From the above postulated pathway for *N*-nitroso carcinogenesis, a series of mechanistic factors may be proposed as being of relevance for modulation of carcinogenic potency, concentrating on the sub-class of dialkylnitrosamines, for which data is most plentiful and internally consistent. These factors include;

1. Facility of α-hydroxylation :

It is first assumed possible that intrinsic chemical facility of α-hydroxylation could be of consequence for carcinogenic

potency. This may be gauged by viewing the process as consisting of abstraction of an α-hydrogen from the nitrosamine by the electrophilic triplet oxygen of the P_{450} cytochrome heme moiety of the hydroxylating enzyme. Facility of α-hydroxylation may be expressed in terms of calculated indices, *viz.* *(a)* the Wiberg bond strength index W_{ch} for the *C-H* bond broken, *(b)* the negative charge Q_h on the α-hydrogen, and *(c)* the frontier orbital factor T_{fo} given by:

$$T_{fo} = C_h/(E_s - E_h) + C_l/(E_l - E_s) \qquad (1)$$

where C_h and C_l are respectively the HOMO and LEMO coefficients of the *α*-hydrogen *1s* orbital, E_h and E_l the HOMO and LEMO energy levels respectively, and E_s the energy level of the singly occupied orbital of the triplet oxygen. The T_{fo} term is a factor in the expression for the frontier orbital interaction energy between the two systems, being ultimately derived from the complete perturbational treatment of Klopman and Salem (1974).

While α-hydroxylation is the chief activating pathway, other routes like *β*-to *w* -hydroxylation are possible, and lead to loss of the potential DNA modifying species since the products can be eliminated from the system in the form of soluble esters like glucuronides. Unlike the *α*-hydroxy product, the *β*-to *w*-hydroxy products are not susceptible to spontaneous decomposition. The chances for activating hydroxylation to occur then may be expressed as the factor F_α, where;

$$F_\alpha = N_\alpha/N_t \qquad (1)$$

where N_α and N_t are respectively the number of hydrogens on the α-carbon and the total number of hydrogens in the nitrosamine which are prone to enzymatic hydroxylation. This assumes that the probability of hydroxylation is the same for all hydrogens in the molecule, since the exact weightages to be assigned are not known.

2. Diazonium ion hydrolysis :

The alkanediazonium ion is prone to hydrolysis after its formation *in vivo*, giving rise to non-carcinogenic products like alcohols. This deactivating hydrolysis competes with the activating route of DNA alkylation. We assume that, on a statistical basis, there is a certain average distance D between the site of formation of the diazonium ion and the site of critical DNA modification. The pseudo-unimolecular hydrolysis reaction follows the rate law

$$C_t = C_o.\exp(-kT) \qquad (2)$$

where C_o and C_t are respectively the concentrations of the ultimate carcinogen when initially formed and when about to modify DNA at the critical site, k is the specific rate constant, and T the time taken to traverse the distance D. T is inversely dependent upon the speed of the diazonium ion in the medium, in turn inversely dependent upon $\sqrt{M_{dz}}$ the square root of the molecular mass, so that

$$T \propto \sqrt{M_{dz}} \qquad (3)$$

or

$$T = \sigma.\sqrt{M_{dz}} \qquad (4)$$

From Eqns. (2) and (3) we get,

$$\ln(C_t/C_o) = -k\sigma.\sqrt{M_{dz}} \quad (5)$$

or $$\ln(C_t/C_o) \alpha 1/\sqrt{M_{dz}} \quad (6)$$

The fraction of diazonium concentration at the moment of DNA modification is thus inversely proportional to the square root of the molecular mass. This factor may be expressed by the index R_{dz}, where

$$R_{dz} = 10/\sqrt{M_{dz}} \quad (7)$$

the numerator 10 being chosen for convenience of representation in the form of numbers between 0.0 and 10.0. The index indicates that larger values of $\sqrt{M_{dz}}$ would lead to lower values of the effective concentration C_t and vice versa.

Another index to be used for estimating the extent of loss due to hydrolysis is the surface area S_{dz} of the diazonium ion in square angstrom units. This is relevant because the greater the surface area, the more prone the species is towards retardation through friction with the surrounding medium, and hence more time elapses before it reaches the target, allowing for more loss of the potential alkylating species. The values of S_{dz} are obtained using the van der Waals radii as proposed by Bondi (1969).

3. O-selective DNA modification :

O-selectivity of DNA alkylation has been earmarked as a factor of relevance for the carcinogenic effect owing to the promutagenic and procarcinogenic properties of *O*-alkylated DNA lesions as opposed to *N*-alkylated ones as described earlier (

Loveless, 1969; Lindahl *et al.*, 1988; Dolan & Pegg, 1985; Huh & Rajewsky, 1986; Scriber & Ford, 1982). It has been proposed by Ford and Scriber, 1990; that *O*-selectivity of DNA alkylation by the diazo-nium ion is related to the S_N1 character of the trnasition state. This S_N1 character has been linked with facility of dissociation of the alkanediazonium ion, given by the enthalpy of dissociation ΔH_d, where

$$RN_2 = R^+ + N_2 + \Delta H_d \qquad (8)$$

The various diazonium ions may be assigned ratings with respect to *O*-selectivity of DNA modification on the basis of the ΔH_d index, where higher values of ΔH_d imply lower levels of *O*-selectivity and vice versa. Experimental observations (Lawley, 1984) on *in vitro* alkylation of DNA by alkylnitrosoureas indicate that the level of *O*-selectivity follows the order *sec*-alkyl > *p*-alkyl > methyl with respect to the alkyl groups presenting in the parent carcinogen. This means that the contribution of *O*-selectivity towards the carcinogenic effect follows this order.

4. Role of DNA repair :

Repair of the *O*-alkylated lesions would be a deactivating or detoxifying pathway detracting from the net carcinogenic effect. Experimental observations furnish repair rates for O^6-alkylguanines in the order *sec*-alkyl < *p*-alkyl < methyl with respect to the alkyl group (Montesano *et al.*, 1979; Pegg & Balog 1979; Pegg & Perry, 1981). This means that the contribution towards retention of the carcinogenic effect would be in the order *sec*-alkyl > *p*-alkyl > methyl, which is the same

qualitatively as that for *O*-selectivity of alkylation. This suggests that branching and complexity of the alkyl group works through two different effects, *viz.* via *O*-selectivity and also via the effects of structure upon repair facility.

5. Role of Target Organ

One of the important factors in determining the relative carcinogenic potency in test animals by *N*-nitroso compound would be its choice of target organ. Upon this target organ selection, when subjected to various routes of administration would reveal their carcinogenic effects and the degree of their carcinogenic potency. The manifestation of this carcinogenic effects in the selected organ is in turn related to their carcinogenic potency of the carcinogens itself. Example of the type of co-relation may be cited here to the remarkable choice of target organ by a number of direct-acting *N*-nitroso compounds upon different route of administration. Chapter VI of this thesis describes in more detail about the choice of target organ by *N*-nitroso compounds.

V.3 Quantification of Factors Determining Potency

A classification scheme is resorted to here, grouping the nitrosamines on the basis of structure of the alkyl groups contained in them. An alkyl group is classified as being *A* type if it does not contain any electron-donating group on its α-carbon. Type *B* alkyl groups have one such electron-donating group, while type *C* alkyl groups have two such groups. Each dialknitrosamine may thus be classified with regard to the type of each of the two alkyl groups contained in it, so that symmetrical nitrosamines may be classified as *AA*, *BB* or *CC* types.

Table.V.4: AM1 calculated values for T_{fo}, W_{ch} etc, of hepatocarcinogenic specific dialkylnitrosamines.

ABBREVIATIONS	F_α	Q_h	T_{fo}	W_{ch}	I_c
1. DMeNA	6/6	0.0673	6.5012	0.9604	2.268
2. MeCyNA	5/5	0.0777	2.5662	0.9460	2.180
3. DCyNA	4/4	0.1114	2.7772	0.9381	1.917
4 DEtNA	4/10	0.0820	1.3277	0.9540	3.201
5. DPrNA	4/14	0.0901	7.4415	0.9820	2.056
6. DBuNA	4/18	0.0811	6.3539	0.9780	1.608
7. DPeNA	4/22	0.0959	5.2034	0.9562	0.589
8. BuPeNA	2/20	0.0951	6.8996	0.9700	1.006
9. DPr^{i}NA	2/14	0.0890	1.2470	0.9670	0.969
10. DHeNA	4/10	0.0930	4.0906	0.9580	-0.048
11. DAeNA	4/14	0.0910	1.6663	0.9670	0.737
12. EtHeNA	4/10	0.0920	1.8572	0.9720	1.807
13. MeEtNA	5/8	0.0935	0.0333	0.9572	2.319

Table. V.5: AM1 calculated values for T_{fo}, W_h etc, of oesophagal specific dialkynitrosamines.

ABBREVIATIONS	F_α	Q_h	T_{fo}	W_{ch}	I_c
1. MePeNA	5/16	0.0598	2.9467	0.9600	2.602
2. MeViNA	4/6	0.0890	0.0301	0.9653	2.638
3. MePhNA	3/8	0.0970	2.8910	0.9622	1.602
4. EtBuNA	4/14	0.0892	7.2176	0.9571	2.114
5. EtViNA	3/8	0.0873	0.0238	0.9632	2.638
6. EtPr^iNA	3/12	0.0901	0.0328	0.9730	1.498
7. MeAlNA	5/8	0.0868	0.0141	0.9650	2.097

Table.V.6: AM1 calculated values for F_{dz}, F_{dz} etc, of hepatocarcinogenic specific dialkylnitrosamines.

ABBREVIATIONS	I_c	M_{dz}	F_{dz}	ΔH_d
1. DMeNA	2.268	43.048	0.1524	0.878
2. MeCyNA	2.180	68.065	0.1212	56.362
3. DCyNA	1.917	68.065	0.1212	92.122
4. DEtNA	3.201	57.075	0.1324	0.311
5. DPrNA	2.056	71.102	0.1186	-3.618
6. DBuNA	1.608	85.128	0.1084	-17.231
7. DPeNA	0.589	99.155	0.1004	-14.203
8. BuPeNA	1.006	85.128	0.1084	-24.107
9. DPr^{i}NA	0.969	71.102	0.1186	3.089
10. DHeNA	-0.048	73.080	0.1170	-80.227
11. DAeNA	0.737	115.019	0.0932	-153.398
12. EtHeNA	1.807	7.080	0.1170	-31.889
13. MeEtNA	2.319	43.048	0.1524	17.016

Table.V.7: AM1 calculated values for M_{dz}, F_{dz} etc, for oesophagal tumour specific dialkylnitrosamines.

ABBREVIATIONS	I_c	M_{dz}	F_{dz}	ΔH_d
1. MePeNA	2.602	43.048	0.1524	-4.138
2. MeViNA	2.638	55.082	0.1347	45.855
3. MePhNA	1.602	105.011	0.0976	55.698
4. EtBuNA	2.114	57.075	0.132	-3.577
5. EtViNA	2.638	55.082	0.1347	37.940
6. EtPr^iNA	1.498	57.075	0.1324	31.485
7. MeAlNA	2.097	43.048	0.152	10.519

As will be seen shortly, this classification is related to the potential for *O*-selectivity of alkylation.

Table V.4 gives the values of the various indices of relevance for facility of α-hydroxylation of oesophagal carcinogens while table V.5, gives this classification for the 13 hepatocarcinogenic dialkylnitrosamines studied here, including values of the various indices of relevance for facility of α-hydroxylation, *viz.* the indices for intrinsic chemical proneness towards this step (the W_{ch}, Q_h and T_{fo} indices), as well as the F_{oh} index for chances of α-hydroxylation. Asymmetrical members are treated simply by assuming that the index values which are representative of the nitrosamine are equal to the arithmetic mean of the values for the two symmetrical dialkylnitrosamines corresponding to each of the two different alkyl groups. Table V.6, presents the values of the indices pertaining to the competition with hydrolysis of the ultimate carcinogen (R_{dz} and S_{dz}), as well as the ΔH_d index for *O*-selectivity of alkylation for hepatocarcinogenicity, while table. V.7 presents the indices for the oesophagal tumours.

It is noteworthy that there is no positive correlation between the potency index I_c and the indices for chemical facility of α-hydroxylation. If anything, an inverse correlation is indicated by the negative values of the Spearman rank correlation coefficients. This trends holds for the 3 members of the *AA* class, as well as for the 5 members of the *BB* class. This observation would lead to the inference that intrinsic chemical facility of α-hydroxylation is not a factor contributing positively towards modulation of parent carcinogenic potency.

Table.V.8: Indices for plotting values of I_c verses F_{oh}, $\log F_{oh}$, F_{dz}.

ABBREVIATIONS	I_c	F_{oh}	$\log F_{oh}$	F_{dz}
1.DEtNA	3.201	0.400	-0.398	0.132
2.DPrNA	2.056	0.286	-0.544	0.119
3.DBuNA	1.608	0.222	-0.654	0.108
4.BuPeNA	1.006	0.200	-0.699	0.108
5.DPeNA	0.589	0.182	-0.740	0.101

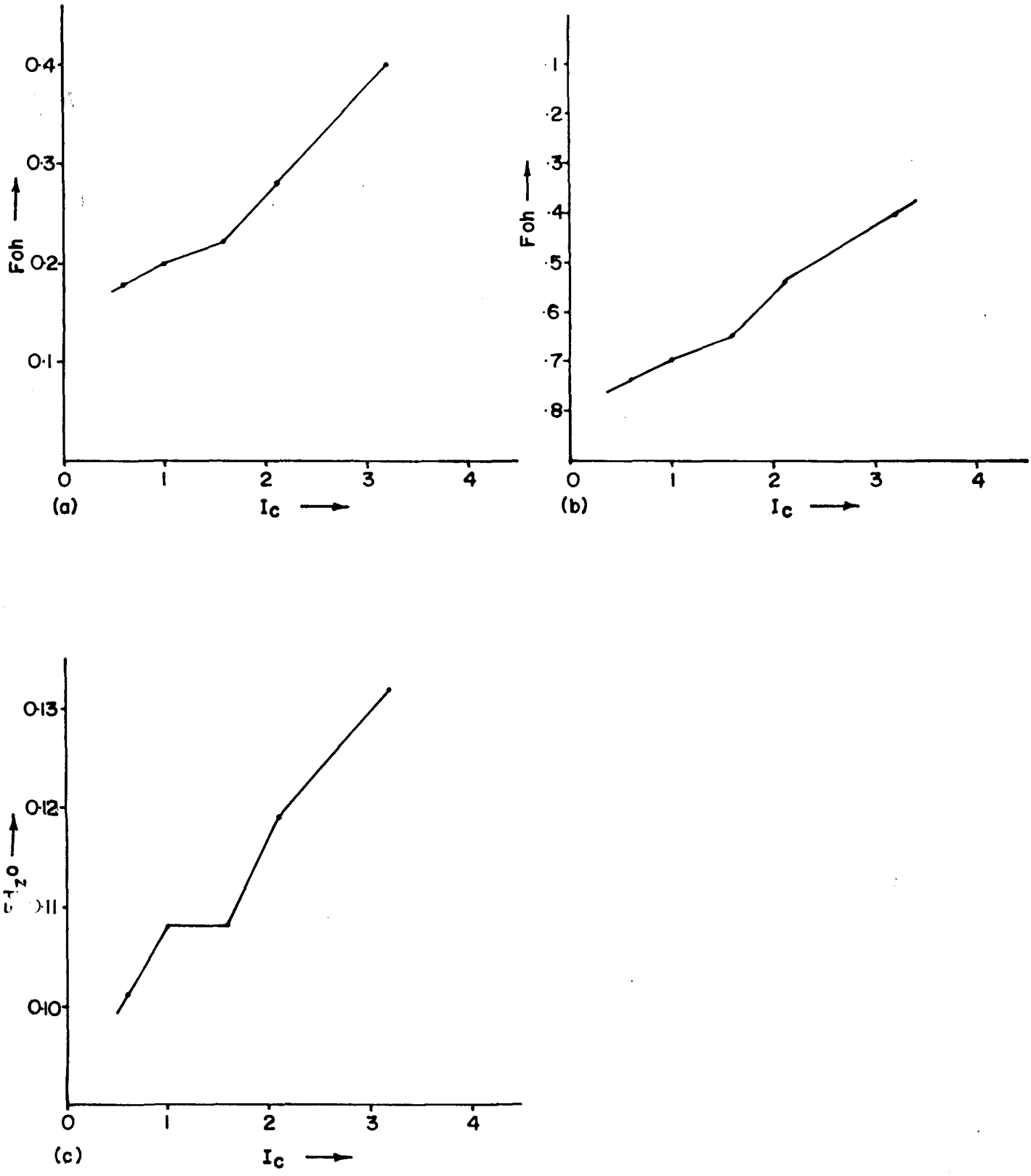


Fig.V.9: Plot of (a) I_c verses Foh (b) I_c vs log Foh (c) I_c vs Fd_z

The indices F_{oh}, R_{dz} and S_{dz} do, however, furnish some positive correlations with potency. It is seen that the best correlation trends are given for the five "*BB*" type nitrosamines. The coefficients of rank correlation between I_c and the theoretical indices F_{oh}, R_{dz} and S_{dz} for these 5 members are all 1.00. This leads to the inference that α-hydroxylation facility and potential for loss of the ultimate carcinogen are factors of direct import for the modulation of relative carcinogenic potency. The plots of potency versus theoretical index are given in Figures V.8 (a) to (d) for the F_{oh}, $logF_{oh}$, R_{dz} and their plotting indices are given in Table.V.9.

The correlation plots for the *AA* types nitrosamines lead to the inference that α-hydroxylation facility needs to be considered along with the potential for hydrolytic loss of the ultimate carcinogen in order to arrive at positive correlations with parent carcinogenic potency. The F_{oh} index considered alone does not contribute anything to the modulation of potency. being equal to 1.00 for all 3 members. The R_{dz} and S_{dz} indices, however, do lead to a correlation coefficient of 1.00.

The values of the ΔH_d index for the various alkyl groups predicts the following order for *O*-selectivity of alkylation : Pr^i > Pe > Bu > Pr > Et > CM > Me. In general terms, the ordering *CC* > *BB* > *AA* is thus seen to hold true for *O*-selectivity. Although there is no direct positive correlation between potency and the *O*-selectivity factor, the effects of this factor upon potency are nevertheless evident, as described below.

Upon examination of the correlation plots for the *AA*, *BB* and

CC type nitrosamines, it is interesting to note that for the F_{oh} index, the *AA* plot lies above the *BB* plot, in turn above the lone position of the *CC* type nitrosamine ($DPr^{i}NA$). This may be interpreted in terms of the effect of *O*-selectivity upon potency, which is in the order *CC* > *BB* > *AA*. In order to arrive at one single plot, it would be necessary to incorporate the *O*-selectivity factor in numerical fashion along with the F_{oh} index. This is the subject of current work here now.

It may be stated that while intrinsic chemical facility of α-hydroxylation is not of direct relevance to the modulation of *N*-nitrosamine carcinogenic potency, the competition between α-hydroxylatiom and deactivating hydroxylation routes proves to be a factor of possible relevance for this. The loss of the ultimate carcinogen through hydrolysis also emerges as a factor of possible import for carcinogenic potency. The factor for *O*-selectivity works indirectly to modulate potency through the ordering *CC* > *BB* > *AA* upon the correlation plots for each separate group. A comprehensive treatment would necessitate the incorporation of all the factors into a single numerical index for potency, which is described and put forward in the following.

V.4. Derivation of Theoretical Potency Index.

This derivation of the potency index K_c assumes that parent carcinogenic potency is directly related to the effective concentration C_{rb} of the *O*-alkylated bases present at the time of DNA replication following administration of a dose C_o of the parent *N*-nitrosamine to the biosystem, C_o being kept uniform for all the cases considered. This concentration C_o may be related to the

experimentally derived potency indices I_c by the following arguments.

Consider two carcinogens *A* and *B*, where *A* ia *n* times more potent than *B*. This means that the values of C_{rb} may be related as given by Eqn. (2) below.

$$C_{rb}(A) = n.C_{rb}(B) \qquad (2)$$

Since *A* is *n* times more potent than *B*, it would require a edian carcinogenic dose that is *n* times less than that for *B*, so that

$$D_{50}(A) = (1/n).D_{50}(B) \qquad (3)$$

From Eqns. (2) and (3), we get

$$C_{rb} \quad \alpha \quad 1/D_{50} \qquad (4)$$

We see that C_{rb} is inversely proportional to $(1/D_{50})$. Now, by appropriate choice of the initial dose C_o and of the units involved, we can put C_{rb} equal to $(1/D_{50})$. This means making the range of C_{rb} and of $(1/D_{50})$ identical, which is useful for making comparisons and examining for correlations, so that

$$C_{rb} = 1/D_{50} \qquad (5)$$

Taking the logarithms of both sides,

$$\log(C_{rb}) = \log(1/D_{50}) \qquad (6)$$

Since $\log(1/D_{50})$ is the form for the experimentally derived potency index I_c as per Eqn. (1), the best form for the theoretically derived potency index K_c which tends to coincide

with the range of values for I_c would be expressible as

$$K_c = \log(C_{rb}) \qquad (7)$$

The various mechanistic factors involved in the theoretical computation of C_{rb} and K_c are dealt with as follows.

1. Concentration of ultimate carcinogen.

The value of C_{rb} would depend upon the effective concentration of the ultimate carcinogen species at the moment of DNA modification. This is treated here as depending in turn upon two factors, *viz.* the probability of α-hydroxylation of the parent *N*-nitrosamine and upon the degree of depletion of the diazonium ion en route from the site of its formation to the site of DNA modification.

The probability of α-hydroxylation occurring in the face of competition from the deactivating β- to ω-hydroxylation routes is expressible in the form

$$F_\alpha = N_\alpha / N_t \qquad (8)$$

where N_α and N_t are respectively the number of α-hydrogens and of the all the hydrogens in the molecule susceptible to enzymatic hydroxylation. Note that an equal probability of hydroxylation has been assigned to each of the alternative hydroxylation routes, since no numerical data is available for assigning proper weightages to each pathway.

The concentration U_o of the ultimate carcinogen at the moment of its formation would be dependent upon the yield of α-hydroxylated product released following adminstration of the uniform dose C_o of the nitrosamine, so that

$$U_o = F_\alpha . C_o \tag{9}$$

Now, this concentration U_o gets depleted in transit through hydrolysis to non-carcinogenic products as it moves from the site of its formation to the site of DNA modification. In order to treat this, it is first assumed on a statistical basis that a certain average distance D exists between the site of formation and the site of critical DNA modification. The time T taken for the diazonium ion to traverse this distance is inversely proportional to the speed of the species in the medium, in turn inversely proportional to the square root of the molecular mass $\sqrt{M_{dz}}$ of the diazonium ion, so that;

$$T \propto \sqrt{M_{dz}} \tag{10}$$

$$\text{or} \quad T = \sigma . \sqrt{M_{dz}} \tag{11}$$

The reaction of hydrolytic loss of the ultimate carcinogen may be now treated assuming a first-order rate law for this pseudo-unimolecular reaction, so that the effective concentration U_t of the ultimate carcinogen at the moment of modifying DNA (ie. at a a time T after its formation) may be given as

$$U_t = U_o . \exp(-kT) \tag{12}$$

where k is the specific rate constant for the reaction. From Eqns. (9), (11) and (12) we get

$$U_t = C_o . F_\alpha . \exp(-k\sigma . \sqrt{M_{dz}}) \tag{13}$$

$$U_t = C_o . F_\alpha . \exp(-\mu . \sqrt{M_{dz}}) \tag{14}$$

2.O-Selectivity of DNA alkylation.

The promutagenic role of the *O*-alkylated bases implies that the relative abundance of formation of these adducts, or the *O*-selectivity of the DNA modification reaction, would be a factor of relevance for the value of C_{rb}. Expressing the relative abundance of *O*-alkylation as the factor Ω, we get

$$C_{rb} = \Omega . U_t \qquad (15)$$

$$\text{or} \quad C_{rb} = C_o . F_\alpha . \exp(-\mu . \sqrt{M_{dz}}) . \Omega \qquad (16)$$

Taking the logarithm of both sides, we get

$$\log(C_{rb}) = \log(C_o) + \log(F_\alpha) + \log(e).(-\mu \sqrt{M_{dz}}) + \log(\Omega) \qquad (17)$$

Remembering that $\log(C_{rb})$ equals the theoretical potency index K_c, and replacing $\log(F_\alpha)$ by the term ϕ, the term $\{\log(e).\mu)$ by τ, and putting w equal to $\{\log(\Omega) + \log(C_o)\}$, we get

$$K_c = \phi - \tau . \sqrt{M_{dz}} + w \qquad (18)$$

Since $\log(C_o)$ is a constant, the term is still representative of the *O*-selectivity factor.

Treatment of Asymmetrical Dialkylnitrosamines

Unlike its symmetrical counterparts, an asymmetrical nitrosamine does not usually have an equal likelihood of α-hydroxylation at both the alkyl groups R_1 and R_2. It may be treated by initially assuming that the two alkyl groups have equal chances of being α-hydroxylated. The concentrations of *O*-alkylated base from each of the groups R_1 and R_2, given as C_{rb}

and C_{rb} respectively, may be expressed separately as per the formulations of Eqns. (16) and (18), where these equations are applied to the two groups

$$\log(C_{rb}') = \phi'' + \tau'.\sqrt{M'}_{dz} + w' \qquad (19)$$

$$\text{and } \log(C_{rb}'') = \phi' + \tau''.\sqrt{M''}_{dz} + w'' \qquad 20$$

The chief difference in this formulation from that of Eqn. (18) is that the ϕ values refer only to one of the two groups, while in Eqn. (18) the α-hydrogens of both groups are considered together. Note also that the alkyl group α-hydroxylated is not the one present in the diazonium ion, expressing the differences by ' & ''.

Upon calculating the values of C'_{rb} and C''_{rb} from Eqns. (19) and (20) by taking the anti-logarithms, we obtain the quantity C_{rb} representative of the nitrosamine as a whole, given by

$$C_{rb} = C'_{rb} + C''_{rb} \qquad (21)$$

from which the potency index K_C may be obtained as

$$K_C = \log(C_{rb}) \qquad (22)$$

If the value of K_C thus calculated does not correspond with the I_C value, the inference to be made within the framework of this model is that the two alkyl groups are not equally prone to being α-hydroxylated. This may be elaborated by calculating the terms K_1 and K'_2 for the alkyl groups R_1 and R_2 respectively, where;

$$K_1 = \log(2C'_{rb}) \quad (23)$$

$$K_2 = \log(2C''_{rb}) \quad (24)$$

If the calculated value of K_C is closer to K_1 than to R_2, and falls in between the two values, this indicates (within the assumptions of the model) that the alkyl group R_1 is more prone to α-hydroxylation than R_2, and vice versa.

V.5. Application of Theoretically Derived Potency Index

We first treat the 5 *BB* type nitrosamines. Because of the similarity of structure of the alkyl groups around the α-carbon, we assume that they all possess the same values of the *O*-selectivity factor and of the hydrolysis factor τ. Taking the most potent member of the class (DEtNA) and the least potent member (DPeNA), the values of ϕ and of $\sqrt{M_{dz}}$ are calculated and inserted into Eqn. (17), making the K_c value equal to the I_c value in order to solve for the yet unknown factor and τ factor. The two simultaneous equations in and τ are then solved. These values of w and τ are then used to predict the potency index K_c for the three other members, *viz.* DPrNA, DBuNA and BuPeNA, after calculating the values of ϕ and $\sqrt{M_{dz}}$ for them. The asymmetrical member BuPeNA is treated as described above for asymmetrical nitrosamines. The values of F_α, ϕ, Γ, w and $\sqrt{M_{dz}}$ are given in table.V.10 along with the values of the experimental and theoretical potency indices I_c and K_c for these 5 nitrosamines under the heading *BB* type, while *AA* types and others alongwith the experimental and theoretical potency indices I_c and K_c for oesophagal carcinogenicity are portrayed in table.V.11 and

Table.V.10: Experimental and theoretical potency index of *"BB"* types dialkylnitrosamines for hepatocarcinogenesis

Abbrev.	I_c	F_α	0	w	t	M_{dz}	$\sqrt{M_{dz}}$	C_{arb}	K_c
1.DEtNA	3.201	2/5	-0.398	10.734	0.945	57.07	7.55	1588.55	3.201
2.DPrNA	2.056	2/7	-0.544	10.734	0.945	71.10	8.43	168.267	2.226
3.DBuNA	1.608	2/9	-0.653	10.734	0.945	85.12	9.22	23.281	1.367
4.DPeNA	0.589	2/11	-0.740	10.734	0.945	91.15	9.95	3.882	0.589
5.BuPeNA	1.006	2/9	-0.653	10.734	0.945	85.12	9.22	23.281	1.067
		2/11	-0.740	10.734	0.945	99.15	9.95	3.882	0.589

Table.V.11: Experimental and theoretical potency data of *"AA"* types and other types of dialkylnitrosamines for hepatocarcinogenicity.

Abbrev.	I_c	F_α	0	w	t	M_{dz}	$\sqrt{M_{dz}}$	C_{arb}	K_c
[A] 'AA' Types :									
1.DMeNA	2.268	3/3	0.00	3.631	0.208	43.04	6.56	185.353	2.268
2.DCyNA	1.917	2/2	0.00	3.631	0.208	68.06	8.25	82.604	1.917
3.MCyNA	2.180	3/3	0.00	3.631	0.208	68.06	8.25	82.604	1.917
[B] Other Types:									
1.DPr^{i}Na (CC)	0.969	1/7	-0.845	9.730	1.098	71.10	8.432	2.383	0.780
2.DHeNA (DD)	-0.048	2/5	-0.398	9.730	1.098	73.08	8.549	0.875	-0.058
3.EtHeNA	1.807	2/5	-0.398	9.730	1.098	57.07	7.555	10.814	1.734
(BD)		2/5	-0.398	9.730	1.098	73.08	8.549	1.143	0.058
4.DAeNA (DD)	0.737	2/7	-0.544	9.73	1.098	115.01	10.73	-0.003	0.594
5.MeEtNA	2.319	3/3	0.000	9.730	1.098	43.048	6.561	208.3	2.319
(AB)		2/5	-0.398	9.730	1.098	57.075	7.555	10.81	1.034

Table.V.12: Experimental and theoretical potency index of oesophagal dialkylnitrosamines.

Name	I_c	F_α	M_{dz}	$\sqrt{M_{dz}}$	0	t	w	C_{arb}	K_c
1.MeViNA	2.63	3/3	43.048	6.557	0.00	0.748	7.543	414.954	2.618
2.MeAlNA	2.097	3/3	43.048	6.557	0.00	0.748	7.543	32.041	1.960
3.MePeNA	2.602	3/3	43.048	6.557	0.00	0.748	7.543	11.138	2.470
4.MPhNA	1.602	3/3	43.048	6.557	0.00	0.748	7.543	13.508	1.431
5.EtViNA	2.638	2/5	57.075	7.549	0.00	0.748	7.543	26.763	2.437
6.EtPr NA	1.498	2/5	55.075	7.549	0.00	0.748	7.543	27.164	1.434
7.EtBuNA	2.114	2/5	57.075	7.549	0.00	0.748	7.543	27.966	2.047

table.V.12 presents the experimental and theoretical index of oesophagal carcinogenic dialkylnitrosamines respectively.

Similarly, for the *AA* type nitrosamines, we initially assume that DMeNA and DCMNA have equal values of the factor and of the factor, to be refined later as described below. Calculating their ϕ and $\sqrt{M_{dz}}$ values, we get two simultaneous equations to be solved for the and the factors. These initial values of are refined by assuming a linear relationship exists between ω and the enthalpy ΔH_d of S_N1 dissociation of the diazonium ion, which had been earlier linked to the *O*-selectivity of alkylation, or

$$w = m.\Delta H_d + c \qquad (25)$$

Now, the rough value for the *AA* type nitrosamines is further assumed to be the arithmetic average of the refined values of for the methyl and cyanomethyl groups, so that

$$w_{AA} = (w_{Me} + w_{CM})/2 \qquad (26)$$

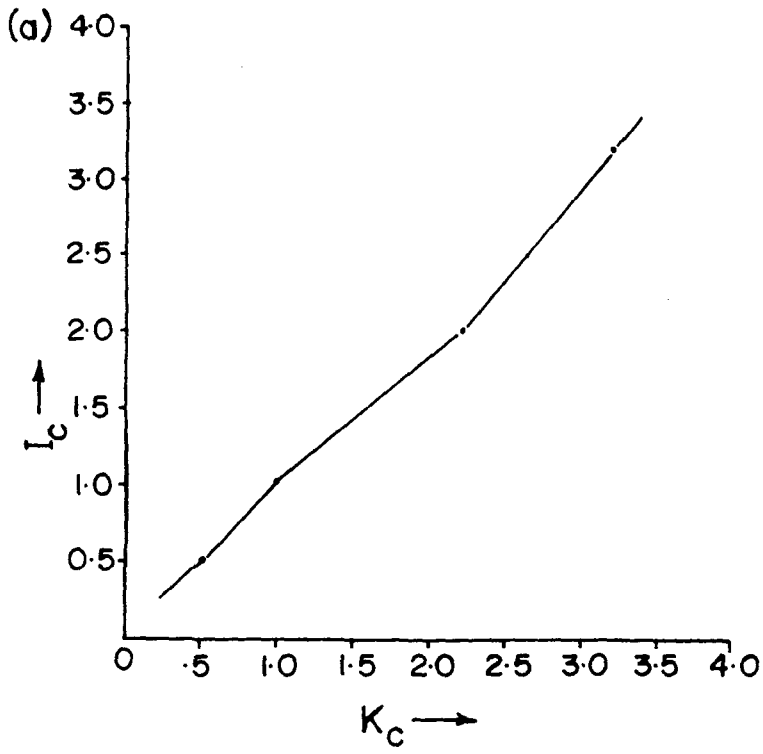
and $$w_{CM} = 2.w_{AA} - w_{Me} \qquad (27)$$

The expressions for w_{AA}, w_{Me} and w_{CM} are now taken together with the value of worked out earlier for the ethyl group, and reference made to the values of ΔH_d as calculated by the AM1 MO method, all put into Eqn. (25), so as to yield three equations in two variables *m* and *c*.

$$w_{Me} = m.(28.42) + c \qquad (28)$$

$$w_{CM} = m.(16.24) + c \qquad (29)$$

$$w_{Et} = m.(13.94) + c \qquad (30)$$



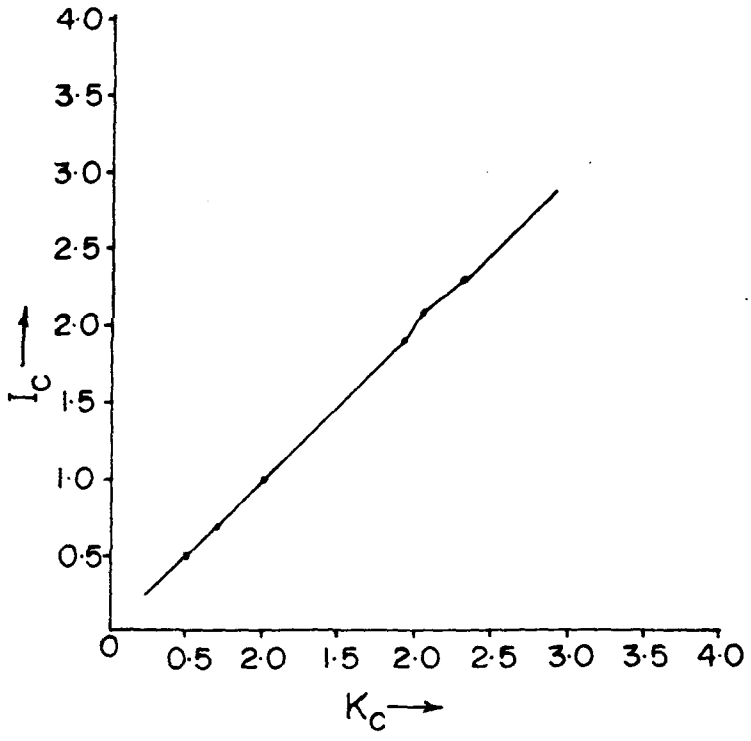


Fig V.13: 11 Plots of I_c verses K_c (a) 'BB' types (b) 'AA' types and others for hepatocarcinogenesis.

The expression for w_{CM} in Eqn. (29) is got from Eqn. (27), and the resultant equation added to Eqn. (28) to eliminate w_{Me}. This leaves two equations to solve for *m* and *c*. Once solved, the AM_1 values of ΔH_d are used to obtain the values for w_{Me} and w_{CM} for the methyl and cyanomethyl cases from Eqn. (25).

Treatment of the *CC* type nitrosamine DPr^iNA is done by first obtaining the value of w_{Pr} from Eqn. (25). This value is taken into Eqn. (17) by coinciding the values of I_c and K_c to solve for the value of w_{Pr}. The plot of experimental potency index I_c verses theoretically derived potency index K_c is given in Figs.V.13.

V.6. Results and Discussions.

In determining the relative carcinogenic potencies of N-nitroso compounds, particularly that of dialkylnitrosamines, various methods like the Iball index, the Wishnok-Archer index, and the use of a varying number of crosses of Andrews and Lijinsky serve a useful guideline to assess the relative carcinogenic potencies of *N*-nitroso compound. In conjunction with this wealth, our mechanistic factors which are incorporated together in the form of a single numerical index for the parent carcinogenic potency generate a reliable carcinogenic potency values of dialkylnitrosamines. It may be worth noted here that the derivation and the application of our theoretical derivation potency index produce a validated result in quantifying the carcinogenic potency values for hepatocarcinogenic specific dialkylnitrosamines. The plot of theoretically calculated potency values K_c verses the experimentally derived relative carcinogenic potency index I_c, indicate

a closely fitting values of *"AA"* and *"BB"* types, which are all hepatocarcinogenic dialkylnitrosamines.

As for the oesophagal specific dialkylnitrosamines, a few of the theoretically derived potency values seem to deviate from that of I_C. This discrepancy between the two may still hopefully be improved by refining the numerical index. Otherwise, all these series of mechanistic factors such as for α-hydroxylation feasibility, for hydrolytic loss of the ultimate carcinogen species, for the *O*-selectivity of DNA alkylation, and the role of target organ are all important parameters to determine the relative carcinogenic potency of the chemical carcinogen *in vivo*. Nevertheless, further research work is being suggested here for those organ-specific carcinogens like that of *N*-nitrosoureas specific for neurotropic effect, whose relative carcinogenic potency have not been worked out as yet.

CHAPTER SIX

RATIONALISATIONS AND PREDICTIONS FOR CHOICE OF TARGET ORGAN(S) IN *N*-NITROSO CARCINOGENESIS

Contents:

VI.1. Choice of Target Organs for N-Nitroso Carcinogenesis

Much labour has been expended on studying the remarkable features of *organspecificity* and *choice of target organ* in *N*-nitroso carcinogenesis. These furnish some of the most fascinating and intriguing aspects of cancer research on NOC. The review articles of Druckrey *et al.* (1967), of Magee (1976) and of Preussmann and Stewart (1984) summarise the current information available at the time on this aspect. Here, however, the well-studied phenomenon of organ specificity *per se* is to be distinguished from that of the choice of target organ for tumourigenesis. The slight distinction between these two may be illustrated by the following:

Many asymmetrical dialkylnitrosamines produce oesophagal cancers in rats regardless of administration route, which persistent effect may be described as truly organotropic or organ specific. Similar also is the case for induction of nervous

system cancers by alkylnitrosoureas. However, the choice of target organ in many cases is dependent upon the administrative route especially for direct-acting carcinogens. For example, *N*-nitrosomethylurea taken orally produces brain and nervous system cancers in rats, but skin cancers when painted on the skin; subcutis cancers when administered by subcutaneous injection and mammary gland cancers when administered direct to the mammary gland. In this thesis more emphasis is laid upon the choice of target organ manifested in a particular case rather than in the genuine organotropic effects. Owing to the abundance of data pertaining to rats, most of the cases referred to here pertain to carcinogenesis in this rodent species. Any general statement in this Chapter regarding choice of target organ may be taken to refer to *rats* unless explicitly specified otherwise, being deduced from the data base available in the review articles mentioned above, to which reference may be made.

The following instances of target organ choice are noteworthy:

(a) Rat liver carcinogenesis by symmetrical DRNA: By far the vast majority of symmetrical DRNA which are active as carcinogens in rats produce tumours of the liver. It is to be noted here that the definition of symmetry as per section I.4 of this thesis (focussing upon identity of the two alkyl groups) proves to be much more efficient in defining the structural criterion for rat hepatocarcinogenesis than the definition adopted by Preussmann and Stewart (1984), where the mere number of carbons in each group is what counts. Out of the 55 symmetrical DRNA (as per the

old definition of symmetry), only 21 (38.1 %) are hepatocarcinogenic in rats. However, out of the 29 DRNA defined as symmetrical by the new definition, 26 or 89.7 % are hepatocarcinogenic in rats. Apart from the liver, other organs may also be affected here, notably the kidneys and lungs.

(b) Rat oesophagal carcinogenesis by asymmetrical DRNA : Asymmetrical DRNA, notably those with one methyl group, and particularly the unsubstituted and non-*C*-oxidised sub-classes, are noteworthy for the manifestation of oesophagal cancers in rats. In many cases this appears to be a genuine organotropic effect independent of administration route. This specificity for oesophagal carcinogenesis by the series of methyl-*n*-alkylnitrosamines continues upto the *n*-heptyl chain for the longer alkyl groups. Here again, the new definition of symmetry proves to be much more efficient in determining the structural criterion for choice of target organ than the old definition of Preussmann and Stewart. For instance, ethylvinylnitrosamine (defined as symmetrical by the old definition) is not hepatocarcinogenic, but a potent oesophagal carcinogen (Druckrey *et al.*, 1967), being correctly defined as asymmetrical by the new definition.

(c) Rat urinary bladder cancers by DRNA with w-oxidised n*-butyl groups:* Along with di-*n*-butylnitrosamine, this class of compounds is noteworthy for induction of urinary bladder cancers in rats as well as other species. The alkyl group apart from the *w*-oxidised *n*-butyl group may range from methyl to *n*-pentyl in the cases tested so far (Suzuki *et al.*, 1981). The type of tumours here arise from the epithelium, and grow to form well-differentiated tumours supported by vascularized connective tissue stroma. Upon

onset of metastasis, tumour invasion extends to the bladder wall itself and to neighbouring organs like the vagina or prostrate.

(d) Hamster pancreatic cancers by DRNA with β-oxidised n-*propyl groups:* A number of DRNA containing a β-oxidised *n*-propyl group are seen to be specific in inducing pancreatic tumours in Syrian hamsters, which sub-class may have two alkyl groups of varying lengths as well (Kruger, 1971; Gingell *et al.*, 1976a; 1976b). The majority of the pancreatic tumours arise in the ducts, mainly in the head of the pancreas (60-70% approx), in the body (20-30 % approx), those in the tail being about 5-10 % (Green *et al.*, 1958; Gullick, 1959).

*(e) Organ-specific effects of methyl-*n*-alkylnitrosamines with long chain* n*-alkyl groups:* When the long chain *n*-alkyl group contains eight or more carbon atoms, tumours of the liver, lung, and urinary bladder are produced in rats (Fischer strain). When the number of carbons is *even-numbered*, urinary bladder and liver cancers predominate, while for those with an *odd-numbered* of carbon atoms in the *n*-alkyl groups, lung cancers are more apparent (Suzuki *et al.*, 1981).

(f) Local tumours for direct-acting carcinogens: Apart from the general class of *N*-nitrosamides, other sub-classes like the α-acyloxyalkyl-, α-hydroxyalkyl-, and α-hydroperoxy-alkylnitrosamines may also be described as direct-acting carcinogens. These produce tumours around the site of application in many cases, *viz.* skin tumours for topical application, subcutis tumours for subcutaneous injection, and forestomach tumours for oral intake. Direct-acting carcinogens can also produce systemic tumours

distant from the site of application depending upon the distance of the tumour site from the administration site, as well as upon the *in vivo* stability of the compound.

(g) Brain, spinal cord and nervous system cancers by alkylnitrosoureas: One of the most remarkable organotropic effects is the induction of brain and nervous system cancers in rats by alkylnitrosoureas, which is largely independent of the route of administration. Altogether, 54 *N*-nitrosoureas have been tested for carcinogenic activity. Out of these, 50 (92.6%) were found to be effective neurotropic carcinogens, inducing nervous system tumours in test animals like mice, rats, Syrian hamsters etc, under various mode of administrations such as oral intake (po), intravenous injection (iv), intraperitoneal injection (ip), or subcutaneous injection (sc) (Druckrey *et al.* 1967; 1970; Druckrey & Landschutz, 1971; Lijinsky & Taylor, 1975; Lijinsky *et al.*, 1980; Zeller *et al.*, 1970; Preussmann & Stewart, 1979). Various class of nitrosoureas that are Considerably effective neurotropic carcinogens include *N*-nitroso-*N*-methylurea, *N*-nitroso-*N*-dimethylurea, *N*-nitroso-*N*-ethylurea, *N*-nitroso-*N*-methylbiuret, *N*-nitroso-*N*-*n*-butylurea etc.

Table VI.1 summerises the above observations regarding choice of target organ by denoting the chief target organ of relevance, the structural type of carcinogen, the total number testing positive for this organ, and the total number of this structural type testing negative for this particular organ. This data was arrived at from a detailed compilation of the tabulations made by Preussmann and Stewart (1984).

VI.2 Mechanistic Factors Involved

The wide range of data summarised in Table VI.1 points to the remarkable selectivity in the choice of target organs for *N*-nitroso carcinogenesis in test animals. Such a large and internally consistent set of findings is surely deserving of a scientific explanation. In the context of this thesis which focusses on mechanistic aspects, the following mechanistic factors emerge as significant and relevant in deciding the choice of target organ.

(a) Animal species and strain: It is noted that an NOC specific for inducing cancer at a particular organ in one species or strain of test animal may not prove to be so for another species or strain. Obviously, the first element of identification of target organ choice has to be with reference to the animal species considered. Species-dependent differences in choice of target organ are evident even upon cursory inspection. In most cases strain differences are not so prominent, but may be noteworthy in some few other cases. As for example, *N*-nitroso-*N*-methylurea does not produce any nervous system tumours on mice and Syrian hamsters under various mode of administrations, while the same compound produced effective tumours of nervous system on brain, and spinal cord in rats, when administered through oral intake (po), intraperitoneal injection, and intravenous injection (Preussmann & Stewart, 1979). Similarly, *N*-nitroso-*N,N'*-dimethyl urea is an effective neurotropic carcinogen on rat *BD* strains, when administered through oral intake, intravenous injection or subcutaneous injection (Druckrey *et al.*, 1967; Jinnai, 1978), but does not produce any nervous system tumours on Syrian hamsters

when administered orally (Hiraki, 1974). While *N*-nitroso-*N*-ethylurea is an effective nervous system carcinogen in rats, mice, and Syrian hamsters, when administered orally, intravenously, intraperitoneally, or subcutaneously (Preussmann & Stewart,1979).

(b) Administration route and dosage schedules: For a genuine organotropic effect, administration route may be a secondary factor. In most cases though, the adoption of different administration routes can lead to the choice of different target organs, especially for direct-acting NOC. The most important administration routes generally employed include the oral intake *(po)*, intravenous injection *(iv)*, subcutaneous injection *(sc)*, intraperitoneal injection *(ip)*, topical application and inhalation *(inhal)*. The effect of administration route upon target organ choice stems in part from the transportation pathway resulting from it, since the pathway may lead directly or indirectly to the target organ.

Oral intake leads to ingestion into the alimentary canal, from which absorption can occur via the hepatic portal vein to the liver, from which transport to various part of the body can occur via the circulatory system following initial metabolism. *Intravenous* administration of NOC leads direct to the heart and lungs via the circulatory system, and then to different parts of the body. Intraperitoneal injection affects first the internal visera within the peritoneal cavity. *Subcutaneous injection* affects first the subcutis and then can lead to distribution via the capillaries of the affected area. *Topical application* initially involves the site of application on the skin. Skin

carcinomas basically may arise from the differentiated squamous epithelial cells or from less differentiated basal cells and pigment cells; as such, skin carcinomas may be described as squamous cell carcinomas or basal cell carcinomas. *Inhalation* would lead first to the nasal cavity and then to the lungs via the trachea. While these succint descriptions of the transportation pathways may seem trivial, they serve to illustrate the point that all these various administration routes would not be without their effects upon the choice of target organ for tumourigenesis.

(c) In vivo *stability of the carcinogen:* DRNA and cyclic nitrosamines as a class are chemically stable and inert, requiring contact with the metabolising (hydroxylating) enzymes system to exert their carcinogenic effects. This pre-supposes the transport of the carcinogens to an organ containing the requisite oxidising enzymes. For the chemically stable *N*-nitrosamines, location of the appropriate metabolising organ along the administration route is of fatal consequence for deciding the carcinogenic effect.

For the relatively unstable direct-acting NOC, relative stability of the carcinogen is not without its effects upon choice of the tumour site. The less stable classes (eg. α-hydroxyalkyl and α-acyloxyalkylnitrosamines), would not be able to extend the region of potential tumourigenesis beyond a certain limit. The more stable alkylnitrosoureas are able to endure transport to more distant sites where they can produce systemic tumours like those of the brain and the nervous system. The

Table VI.1 Summarises the choice of target organs by denoting the target organ, the number tested, the route of administration, the number positive on thetarget organ, the number positive other than the target organ, and percentage positive on target organ.

No.	Class	No. tested	Route	Organ	T_{org}	N_{org}	% T_{org}
A.	**INDIRECT-ACTING NOC.**						
1.	Symmetrical DRNA*	30	po	liver	22	2	73.33
2.	*C*-oxidised sym DRNA	7	po	liver	5	2	71.43
3.	Asymmetrical**	56	po	eso	38	18	67.86
4.	*β*-oxidised *n*-propyl	8	po/sc	pancreas	3	5	37.50
5.	*w*-oxidised *n*-butyl	8	po	bladder	7	5	58.33
6.	Methy-*n*-alkyl(even)	8	po	eso	5	3	62.50
7.	Methyl-*n*-alkyl(odd)	5	po	eso	3	2	60.00
8.	Trialkylnitrosourea	1	po	lung	1	0	100.00
B.	**DIRECT-ACTING NOC.**						
1.	Alkylnitrosoureas	54	po	alim.canal	20	34	37.04
		54	sc	subcutis	9	45	16.66
		54	po/iv	neurons	11	43	20.37
		54	top	skin	22	32	40.74
2.	*N*-nitrosoguanidines	11	po	alim.canal	3	8	27.27
		11	sc	subcutis	5	6	45.45
3.	Urethanes	17	po	alim.canal	14	3	82.35
4.	Nitrosamides proper	18	po	alim.canal	4	14	22.22
		18	sc	subcutis	3	15	16.66
5.	*α*-oxidised nitrosamines	7	sc/po	subcutis forestomach	6	1	85.71

* Seven of them are negative on po in liver.

** Excluding *β*-oxidised *n*-propyl, *w*-oxidised *n*-propyl, oxidised symmetrical DRNA, and *α*-acyloxyalkylnitrosamines.

effect of *p*H upon stability of direct-acting NOC may be mentioned here, which would be of particular relevance for choice of target organ along the alimentary canal following oral intake, since *p*H changes may be dramatic upon passing from one segment of the canal to the other (eg. from stomach to duodenum).

(d) Chemical structures of the carcinogen: Two distinct features of the chemical structure enter into the picture here, *viz.* that of the *functional group* involved and that of the *alkyl group(s)* involved. The functional group determines the sub-class to which the NOC belongs, (eg. nitrosamines, nitrosoureas, nitrosourethanes, nitrosoguanidines etc.), which in turn is of vital importance for deciding choice of target organ, as shown in Sec. VI.1 and Table VI.1 above. The structure of the alkyl group(s) is also important here. Differences in identity between the two alkyl groups of the dialkylnitrosamines may spell the difference between liver and oesophagal carcinogenesis in rats. Oxidation at the β-carbon of the *n*-propyl group and the ω-carbon of the *n*-butyl group in dialkylnitrosamines indicates the choice towards pancreatic cancers in Syrian hamsters and bladder cancers in rats respectively.

(e) Presence and distribution of oxidising enzymes: This applies primarily to indirect-acting *N*-nitroso compounds. It is obvious that only those organs and tissues possessing the requisite α-hydroxylating enzymes would be expected to manifest a carcinogenic effect *within* those organs. Here, a distinction may be made between *direct* and *indirect* metabolism of nitrosamines (a new deduction connected with this thesis, not to be confused

with the distinction between the direct and indirect-acting *N*-nitroso compounds). *Direct metabolism* refers to the generation of a metastable proximate carcinogen followed by generation of the unstable ultimate carcinogen directly from the substrate in one step within the *same* organ or tissue. Direct metabolism thereby refers primarily to the α-hydroxylation mechanism. A consequence of direct metabolism is that the organ site of metabolism must necessarily be also the site of DNA modification by the ultimate carcinogen, owing to the relatively high instability of the product of direct metabolism, *viz.* the α-hydroxylated derivative.

Indirect metabolism refers to a two-stage process, the first of which is the enzymatic formation of relatively stable hydroxy, oxo, or carboxy derivatives at carbon positions other than the α-carbon. Because of the stability of the products obtained, these metabolites may be *transported* from one organ to the other. Upon reaching another organ with the requisite α-hydroxylating enzymes specific for these oxidised substrates, the second stage may occur, involving further metabolism via α-hydroxylation to yield the ultimate carcinogen, which then goes on to modify DNA in the second organ.

Much study has gone into the capacity of various organ tissues for metabolising nitrosamines (Mirvish *et al.*, 1986; 1988). The rat and human liver are a rich store house of oxidase enzymes, and liver microsomes have the ability to oxidise hepato-carcinogenic nitrosamines (Ji *et al.*, 1988) as well as oesophagus specific nitrosamines (Huang *et al.*, 1991). The oesophagus of

rats and humans has a capacity to oxidise the oesophagal carcinogen *N*-nitroso-methyl-pentylamine (Huang *et al.*, 1990; 1991; Mirvish *et al.*, 1987; 1992). These individual capacities of various organs to metabolise various substrates may indeed be a key factor determining organotropy and choice of target organ.

A Model : In order to explain the organotropic effect at organs in the rat other than the liver, recourse has to be made to the following suppositions. The rat liver microsomes may be expected to contain α-hydroxylase enzymes which are specific only for *hepatocarcinogenic* (symmetrical) dialkylnitrosamines. This model assumes that any metabolism of oesophagal (asymmetrical) dialkylnitrosamines in the rat liver would have to be of the *indirect* type, presumably at the β-to w-positions (Mirvish *et al.*, 1985), which would then be followed by transport of these stable metabolites to the oesophagus. In the oesophagus, *direct* metabolism (α-hydroxylation) of the transported metabolites could then occur, assuming the existence in the oesophagus of α-hydroxylase enzymes specific for these transported metabolites. In this way, the organotropy of hepatocarcinogenic and oesophagus specific dialkylnitrosamines may be explained through the following suppositions:

1. Direct metabolic α-hydroxylation occurs within the liver itself only for hepatocarcinogenic (symmetrical) nitrosamines.
2. Indirect metabolism of oesophagal carcinogens (leading to stable transportable metabolites) could conceivably occur within the liver followed by transport to the oesophagus.
3. Direct metabolism for the stable metabolites of oesophagal carcinogens could than occur in the oesophagus itself.

This implies a wide variety and high substrate-specificity of the oxidase enzymes involved, which apparently include those with an ability to distinguish between symmetrical and asymmetrical substrates. The character and distribution of these enzymes as proposed by this model may be summarised as follows:

1. α-hydroxylating enzymes for symmetrical substrates, found in the liver.
2. β-to w-oxidising enzymes responsible for the indirect metabolism of asymmetrical substrates, also found in the liver.
3. Absence in the liver of the α-hydroxylating enzymes which recognise asymmetrical substrates.
4. Presence of the α-hydroxlating enzymes in the oesophagus responsible for the direct metabolism of stable metabolites derived from the indirect metabolism of asymmetrical substrates (like β- to w-oxidised metabolites).

(f) Presence and distribution of DNA repair systems: Carcinogenesis by NOC and alkylating agents is known to involve the critical alkylation of DNA bases. As such, persistence of these genotoxic alkylated DNA lesions in the tissues and organs would be expected to be a contributory factor towards the carcinogenic effect. Thus, it may be surmised that the organotropic effects of these carcinogens might well be related to the *presence and distribution* of the DNA repair enzymes in various organs system. Organs with a poor or error-prone repair capacity may be expected to be more susceptible to the carcinogenic effects of alkylation, and vice versa.

It has been reported that a number of DNA repair enzymes operate in cell lines drawn from bacteria (*E. coli*) as well as from various organs or tissues of different animal and mammalian species. These include the base-excision activity of DNA ***glycosylases*** that has been demonstrated in bacteria for N^3-MeAd (Laval, 1977), for N^7-MeGu (Chetsanga & Lindahl 1979; Laval *et al.*,1981), and the nucleotide-excision activity by apurinic endonucleases in bacteria (Pierre & Laval, 1980). In mammalian cells, evidence for N^3-MeAd glycosylase was reported in human lympoblastoid cells extracts for the repair of N^3- and N^7-alkypurines (Ishiwata & Oikawa, 1979; Brent, 1979). Glycosylase activity for N^3-MeAd was also identified and purified from rat liver nuclei (Cathcart & Goldthwait, 1981). From human placenta (Dosangh *et al.*, 1994), a well-characterized DNA repair enzyme (3-methyladenine-DNA-glycosylase) also capable of removing N^6-ethenoadenine was identified and purified (Singer *et al.*,1992). The general mechanism for repair of N^3- or N^7-alkylpurines (whether spontaneous depurination or enzyme mediated glycosylase activity) is base-excision, involving hydrolytic cleavage of the glycosyl C-N bond of the modified nucleotide. However, the direct relevance for carcinogenesis of glycosylase repair of N^7-alkylguanines is doubtful, since these lesions seem to be innocuous for the cancer process (Chetsanga & Lindahl, 1979; Laval *et al.*, 1981; Pierre & Laval, 1980). Lesions of direct consequence for carcinogenesis and mutagenesis include *O*-alkylated bases like O^6-alkylguanine and O^4-alkylthymine, which are not repaired by glycosylases but by a different group of enzymes called alkyltransferases.

Alkyltransferase enzymes like O^6-alkylguanine-DNA-alkyltransferase (AAT) that accept the alkyl group on to a cysteine residue in the enzyme have been identified and purified both in mammalian cells (Olsson & Lindahl, 1980) and in bacterial cells (Schendel & Robins, 1978). The bacterial alkylguanine-DNA-alkyltransferase is a *37 Kd* protein coded by the *ada* gene with its activity located at the *18 Kd* fragment towards the carboxy terminus (Ahmmed & Laval, 1984), while that of rat AAT weighs about *18.5 to 20.0 Kd* (Dolan *et al.*, 1984; Gerson *et al.*, 1986). The alkyltransferase located in human cells and tumour tissues has been measured (Wu *et al.*, 1987) and reported to be *21 & 24 Kd* (Singer *et al.*, 1981). In human normal and tumour tissues, this O^6-methylguanine-DNA-methyltransferase gene *MGMT* seems to carry *mRNA* (Citron *et al.*, 1992). A number of workers like Bertini *et al.* (1990), Potter *et al.* (1991) and Wilkinson *et al.* (1991) reported the increasing activity of O^6-alkylguanine-DNA-alkyltransferase in rat liver due to interferon inducers, while various groups of workers (Frosina & Laval, 1987; Morohoshi *et al.*, 1989; Pegg & Wiest, 1983; Rahden-Staron & Laval, 1991; Shiraishi *et al.*, 1992; Chang *et al.*, 1993) detected the activity of O^6-methylguanine-DNA-methyltransferase in rat hepatoma cells.

The activity of O^6-methyl-DNA-methyltransferase was also detected and well studied in human cells (Yarosh *et al.*, 1984), in rat kidney, lung, liver (Pegg & Wiest, 1983; Cooper *et al.*, 1978), and in rat mammary epithelial cell (Dutta-Choudhury *et al.*, 1991; Fong *et al.*, 1990). Yarosh (1985) has presented the comparative data on the expressions of O^6-MeGMT activity in various tumour

cells. Apart from this, the active site and complete sequence of the so-called suicidal methyltransferase that counters the damage due to the alkylation mutagenesis has also been discovered and deciphered (Demple *et al.*, 1985).

The relevance of repair of these *O*-alkylated lesions, especially of O^4-alkylthymines (owing to their slowness of repair rate), for initiation of cancer seems to be beyond reasonable doubt, being attested to by the wealth of information on the good correlations observed between the tumour incidence and the persistence of these lesions in tissues and organs of rodents treated with alkylating carcinogens and NOC. The results of single dose, multiple dose and chronic exposure regimes involving administration of methylating NOC to rats support the procarcinogenic role of O^6-methylguanine by affording correlations between tumour incidence and persistence of this lesion in organ tissues (Pegg & Wiest, 1983; Pegg, 1977; O'Connor, 1981). Similar correlations have been obtained for O^4-methylthymine in rat tissues (Richardson *et al.*,1985; Friedberg *et al.*, 1979). A greater likelihood of the O-alkyl pyrimidines (rather than O^6-alkylguanines) being a causative agent in carcinogenesis has been proposed by Singer (1980), drawing upon the greater *in vivo* stability and resistance to repair of these lesions. As such, it may be easily deduced that the presence and distribution of these alkyltransferase enzymes should be a pertinent factor for deciding the organ site for tumourigenesis. Organs and tissues rich in alkyltransferase activity or poor in error-free repair may be expected to possess a greater intrinsic susceptibility towards tumour development, and vice versa. It may be therefore

possible to help predict/explain the choice of target organ and their organ specificity with the existing data available so far with respect to presence and distribution of the DNA repairing enzymes alkyltransferase.

VI.3 Mechanistic Models

Drawing from the above factors described in Sec. VI.2, a mechanistic model may be constructed to furnish rationalisations and predictions regarding choice of target organ for NOC carcinogenesis. Following are two mechanistic models framed to help explain or predict the choice of target organ in animals carcinogenesis by indirect-acting and direct-acting NOC respectively:-

Indirect-acting NOC

The following three mechanistic factors are invoked to create a model for choice of target organ in indirect-acting NOC:

1. Role of administration route: For indirect-acting NOC, the administration route should ensure transport to an organ where oxidative metabolism can occur (direct metabolism). The preceding section outlined some common administration routes and the areas/organs reached by them. Failure of the administration route to arrive at an appropriate metabolising organ may even result in failure of NOC to manifest carcinogenicity. For those NOC where indirect metabolism is necessary, further transport is required to another organ where indirect metabolism can take place.

2. Role of organ(s) of oxidative metabolism: For NOC which act through direct metabolism only, the organ should possess the α-hydroxylase enzyme system specific for the substrate. For NOC

which require indirect metabolism, the primary organ should possess hydroxylating enzymes specific for action on carbons of the substrate other than the α-carbon. Furthermore, the organ of oxidative metabolism should possess facile transport access to the organ of critical DNA modification.

3. Role of organ of DNA modification: For NOC acting through direct metabolism, the organ of critical DNA modifications is the same as the organ where oxidative metabolism occurs. For NOC involving indirect metabolism, this organ should possess α-hydroxylase enzyme systems specific for the metabolised substrate which arrives from the organ of primary metabolism. Moreover, the following characteristics should be present:

(a) Low repair capacity: For the alkylated DNA lesions to persist, the organ cells and tissues should not have an efficient capacity for repair of these lesions. This means that the level of alkyltransferase activity should be low, inefficient, error-prone or absent altogether. The capacity for repair of O^6-methyl-guanine in human tissues follows the order: liver > kidney > colon > oesophagus > lung > brain (Den Engelse *et al.*, 1985; Graftstrom *et al.*, 1983; Wiestler *et al.*, 1984; Myrnes *et al.*, 1983), indicating that the alkyltransferase activity varies from tissue to tissue and organ to organ for the same animal or species to species (Hall *et al.*, 1985; Maru *et al.*, 1982; Craddock & Henderson, 1984; Bamborschke *et al.*, 1983). The repair activity of O^6-MeGMT also varies during foetal and neonatal development (Craddock *et al.*, 1984).

(b) High cell and DNA turnover: Since the genetically encoded information for cancer can be perpetuated and carried on only if DNA replication occurs, the organ of DNA modification should also be characterised by a high rate of cell division and multiplication. Cells which cannot divide or multiply can never become cancerous, eg. the neurons of the brain. Certain procedures like partial hepatectomy stimulate cell division and thereby also pave the way for more facile manifestation of carcinogenic activity. Stem cells have the distinctive capacity of self-renewal to maintain the population size, which then differentiate to form progenitor cells that will ultinately mature to yield functional cells incapable of further division. The balance between cell renewal and cell loss is normally maintain in most mature organs and tissues, which balance becomes disturbed with neoplastic transformation (Farmer & Walker, 1984).
(c) Low immunological defense: The host immune system acts as a defense for prevention of tumour cell growth and spread. As long as this system remains strong enough to withstand the attack of tumourous cells, cancer may be prevented or at least delayed. The breakdown of immunological defenses in the organ of DNA modification could well mean that the way is clear for cancer to manifest itself. As such the importance of immunity to tumours (cancers) is the introduction of *immunotherapy*, which serves as an immunological means to detect tumours and to stimulate the immune system to destroy tumour cells. The incidence of tumours production and spread within an organ or tissue is thus well checked by the immunosurveillance. Thus, to protect from tumour invasion on normal cells, we have tumour specific immune system

like the *T-lymphocytes* and the *Natural killer cells*. The varying distinction between the two is that, T-lymphocytes would kill only the tumours of the same antigen of the correct MHC antigen having genetic restriction, while the natural killer cells have no genetic restriction, which would kill the most *in vitro* grown target cells (Beverley, 1986).

Direct-acting NOC

The following three basic factors constitute the model for explaining choice of target organ in direct-acting NOC:

1. Role of administration route: Since direct-acting NOC decompose spontaneously to yield the ultimate carcinogen directly, the administration route employed should ensure transport directly to the site of critical DNA modification, *viz.* the site of tumourigenesis. Any administration route which cannot ensure this would be abortive.

2. Role of in vivo stability: Different groups of direct-acting NOC are characterised by different *in vivo* stabilities (Lyngdoh, 1994). Higher *in vivo* stability would ensure the possibility of transport to organ(s) further removed from the site of administration. Low *in vivo* stability would mean that the scope is left for carcinogenicity to be manifested only locally around the site of administration. In general, for any one alkyl group, the order of stability with respect to functional group type is alkylnitrosourea > alkylnitrosonitroguanidine > α-hydroxyalkyl-nitrosamine.

3. Role of organ of DNA modification: The aspects under this heading are exactly the same as those covered earlier for indirect-acting NOC.

VI.4 Application of Models

The above models of Sec. VI.3 are now applied to provide rationalisations for the following examples of organ specificity or choice of target organ:

1. Rat hepatocarcinogenesis of symmetrical DRNA

Table VI.2 summarises the available data on liver carcinogenesis in rats of all strains by denoting the structural type of carcinogen, the total number tested, the number producing liver tumours, and the percentage of liver tumour incidence. It can be seen that the class of symmetrical DRNA (with their oxidised derivatives) is the most specific class for induction of rat hepatocarcinogenesis. Quoting the most commonly used oral administration route (po), the three factors of the mechanistic model are applied as follows.

The oral administration route leads to transport into the alimentary canal. The observation that no tumours arise here is indicative of the absence of the requisite metabolising enzymes in any segment along the alimentary canal (at least upto the large intestine). Absorption of the administered carcinogen takes place presumably along the small intestine and finally into the liver.

The liver is the organ where the DRNA is metabolised, presumably by direct metabolism through α-hydroxylation of the substrate. This assumes the presence in the liver of α-hydroxylase systems which can recognise symmetrical DRNA (and their *C*-oxidised derivatives) as specific substrates.

Table VI.2 Summarises Liver as the choice of target organ by denoting the target organ, the structural type of carcinogen, the total number tested, number positive, and percentage positive.

Organ	Structural type	No.tested	No.+ve	% +ve
LIVER	**1.SYMMETRICAL**	**30**	**21**	**70.00**
	(a) Unsubstituted	14	7	50.00
	(b) *C*-Oxidised	7	5	71.43
	(c) *C*-substituted	9	6	66.66
	2.UNSYMMETRICAL	**118**	**37**	**31.36**
	(a) Unsubstituted	53	22	41.51
	(b) *C*-Oxidised	47	15	31.91
	(C) α-acyloxyalkyl	7	0	0.00
	(d) *C*-substituted and others	11	2	18.18
	3. CYCLIC NITROSAMINES	**71**	**31**	**43.66**
	(a) Unsubstituted	15	8	53.33
	(b) Ring C-Oxidised	15	4	26.67
	(c) Ring C-substituted	19	4	21.05
	(d) Ring with hetero-atoms	23	15	65.22
	4. DINITROSAMINES	**11**	**3**	**27.27**
	5. N-NITROSAMIDES	**100**	**9**	**9.00**
	(a) N-nitrosourea	54	5	9.26
	(b) N-nitroso-nitroguanidines	11	3	27.27
	(c) N-nitrosamides Proper	18	1	5.56

Table VI.3 Summarises Oesophagus as the choice of target organ by denoting the target organ, the structural type of carcinogen, the total number tested, number positive, and percentage positive.

Organ	Structural type	No.tested	No.+ve	% +ve
OESOPHAGUS				
	1. SYMMETRICAL	30	5	16.66
	(a) Unsubstituted	14	2	14.29
	(b) C-Oxidised	7	3	42.86
	(c) C-substituted	9	0	00.00
	2. ASYMMETRICAL	118	50	42.37
	(a) Unsubstituted	53	38	71.69
	(b) C-Oxidised	47	9	19.15
	(c) α-acyloxyalkyl	7	0	0.00
	(d) C-substituted and others	11	3	27.27
	3. CYCLIC NA	71	27	38.03
	(a) Unsubstituted	15	6	40.00
	(b) Ring C-Oxidised	15	1	6.67
	(c) Ring C-substituted	19	13	68.42
	(d) Ring with hetero-atoms	23	7	30.43
	4. DINITROSAMINES	11	8	72.73
	5. N-NITROSAMIDES	100	12	12.00
	I. N-nitrosourea	54	3	5.56
	(a) Dialkylureas	20	3	15.00
	(b) N-nitrosourethanes	17	8	47.06
	(c) N-nitrosamides Proper	18	1	5.56

The liver is also the organ for critical DNA modification followed by tumour induction. This means that the liver tissues have relatively low capacity for repair of the critical alkylated lesions. Conversely, it is also a possibility that the amount of genotoxic damage is too large for the repair facility to cope with, owing to the relatively large amounts of the parent carcinogen absorbed into the liver. It may also be presumed that the immunological defense system in the liver fails to cope with the population of cancerous cells arising from the DNA damage.

2. Rat oesophagal carcinogenesis by asymmetrical DRNA

Table VI.3 summarises the available data on oesophagal cancers in rats of all strains, giving the structural type of carcinogen, the total number tested for each type, the number yielding oesophagal cancers, and the percentage of oesophagal cancer incidence. It is immediately evident that the asymmetrical DRNA as a class are most effective and specific for the induction of oesophagal cancers in rats. For the purpose of differentiation, asymmetrical DRNA are subdivided into the unsubstituted, *C*-oxidised, α-acyloxyalkyl, *C*-substituted, and very long alkyl chain subgroups. It is seen that the unsubstituted subgroup is the most specific for induction of oesophagal cancers. Here again, the oral administration route is the most commonly used. The three features of the mechanistic model are now applied as follows.

The oral administration route leads to transport along the alimentary canal. Considering the oesophagal specificity of asymmetrical DRNA, it is a moot question as to whether this might be due to metabolism in the oesophagus itself as the carcinogen

passes through the canal. Two factors might appear to rule this out. The first is the time factor, which allows little time for sufficient accumulation of the carcinogen as it passes through propelled by peristalsis with the food or water medium. The second factor is the observation that oesophagal carcinogenicity of some asymmetrical dialkylnitrosamines appear to be independent of adminstration route, eg. as is the case for methyl-*n*-pentylnitrosamine which produces oesophagal cancers through both oral and subcutaneous routes (Druckrey *et al.*, 1967). Both oral and intravenous administration of ethyl-*n*-butylnitrosamine produce oesophagal cancers in BD rats (Druckrey *et al.*, 1967). Similar also are the cases for ethyl-4-picolylnitrosamine and ethylvinyl-nitrosamine (Druckrey *et al.*, 1967). As such, one would have to point towards the *liver* as the primary organ for initial metabolism, which appears to be of the *indirect* type.

In the liver, it would appear that no α-hydroxylation takes place because of the absence of the appropriate enzymes specific for asymmetrical DRNA substrates. Instead, metabolism could occur via formation of β- to ω-oxidised products which are then transported to other organ(s) of the body by the circulatory system, including the oesophagus. This presupposes the presence of β- to ω-oxidising enzymes in the liver, which act upon the asymmetrical DRNA substrates.

The oesophagus is the site of secondary metabolism (α-hydroxylation) of the stable metabolite(s) obtained from the liver, yielding the ultimate carcinogens (diazonium ions). This would mean that the oesophagus should contain the α-hydroxylase enzymes specific for the liver metabolite substrates.

The oesophagus is also the site of critical DNA modification. Being as such the organ for tumourigenesis, the oesophagus would be expected to be characterised by low DNA repair capacity, high cell and DNA turnover rate and low immune defense profile.

3. Neurotropic carcinogenesis of nitrosoureas

Table VI.4 summarises the available information on the brain and nervous system as the target organ by denoting the structural type of carcinogen, the total number tested, the number yielding neurotropic tumours, and the percentage incidence of neurotropic tumours. It is noted that the class of nitrosamines (both dialkyl and cyclic) are conspicuous by the virtual absence of ability to induce neurotropic tumours, regardless of administration route. As such, Table VI.4 gives information only for the class of *N*-nitrosamides, including nitrosoureas, nitroso-urethanes, nitrosoguanidines, and others. From all these sub-classes, the nitrosoureas are seen to be prominent as neurotropic agents in rats. This is independent of the administration route.

The three factors of the mechanistic model are now applied to rationalise the above findings.

Although the neurotropic activity of nitrosoureas appears to be independent of the administration route, choice of other target organs is closely associated with the administration route employed. This is true for induction of skin tumours in mice by topical application (IARC Monograph No.17, 1979), subcutis tumours through sc injection, and alimentary canal tumours in rats by oral administration. All of these other effects are more or less local-acting and are dealt with later.

The neurotropic activity of nitrosoureas being independent of

Table VI.4 Summarises Nervous system as the choice of target organ by denoting the target organ, the structural type of carcinogen, the total number tested, the number positive, and the percentage positive.

Organ	Structural type	No.tested	No.+ve	% +ve
NERVOUS SYSTEM				
	3. CYCLIC NA	71	2	2.82
	(c) Ring C-substituted	19	2	10.53
	2. N-NITROSAMIDES	100	26	26.00
	1. N-nitrosourea	54	5	9.26
	(a) Monoalkylureas	33	14	42.42
	(b) Dialkylureas	20	12	60.00

administration route seems to be associated rather with some intrinsic property of these compounds themselves. One such property is the relatively high *in vivo* stability as compared to other direct-acting NOC such as nitrosoguanidines, α-acetoxy-methylnitrosamines, and nitrosourethanes (all of which induce only local-acting tumours, not systemic). This stability would be able to ensure sufficient transport to regions fairly far removed from the site of administration, viz. the brain and nervous system.

In addition to the stability factor, another factor of relevance for neurotropic activity would be the ability to penetrate the blood-brain barrier. This would be associated with a high degree of lipophilicity, which would be expected to be characteristic of any central nervous system specific agent.

Since the brain and nervous system are the target organs for the neurotropism of nitrosoureas, one would expect that these organs would display the above-mentioned characteristics typical of target organs, viz. low DNA repair capacity, high cell and DNA turnover rate, and low immunological defense profile. It is noteworthy that brain tissues rank lowest among the various human organ tissues tested for capacity to repair O^6-MeG (Wiestler *et al.*, 1984; Graftstrom *et al.*, 1983; Den Engelse *et al.*, 1985; Myrnes *et al.*, 1983), while rat brain tissue ranks even lower (Wiestler *et al.*, 1984). With regard to cell turnover rate, although the neurons are characterised by a zero turnover rate, the brain glial tissue is fully capable of undergoing cell renewal and is the actual part of the nervous system affected by tumourigenesis.

Table VI. 5 Summarises alimentary canal system as the choice of target organ by denoting the target organ, the structural type of carcinogen, the total number tested, the number positive, and the percentage positive.

Organ	Structural type	No.tested	No.+ve	% +ve
ALIMENTARY CANAL SYSTEM	1. **SYMMETRICAL**	30	4	13.33
	(a) *C*-Oxidised	7	3	42.86
	(b) *C*-substituted	9	1	11.11
	2. **UNSYMMETRICAL**	118	20	16.95
	(a) Unsubstituted	53	11	20.75
	(b) *C*-Oxidised	47	3	6.38
	(C) α-acyloxyalkyl	7	6	85.71
	(d) *C*-substituted	11	0	0.00
	3. **CYCLIC NA**	71	12	16.90
	(a) Unsubstituted	15	2	13.33
	(b) Ring *C*-substituted	19	8	42.11
	(c) Ring with hetero-atoms	23	2	8.70
	4. **DINITROSAMINES**	11	3	27.27
	B. **N-NITROSAMIDES**	100	48	48.00
	1. *N*-nitrosourea	54	24	44.44
	(a) Monoalkylureas	33	14	42.42
	(b) Dialkylureas	20	10	50.00
	(c) Trialkylureas	1	1	100.00
	2. *N*-nitrosourethanes	17	16	94.12
	3. *N*-nitroso-nitroguanidines	11	4	36.36
	4. *N*-nitrosamides proper	18	3	16.67

It is a pertinent question as to why indirect-acting NOC are not neurotropic. The reason may be hypothesised to lie in the following factors: *(a)* Absence of requisite metabolising enzymes in the tissues of the brain and nervous system, and *(b)* inability of the liver-produced metabolites to penetrate the blood-brain barrier. For the other classes of direct-acting NOC which are not neurotropic, the explanation may lie in their low *in vivo* stability which enables them to exert their carcinogenic effects only locally.

4. Alimentary canal carcinogenesis in mammals

Table VI.5 summarises the available information on the alimentary canal (various segments excluding the oesophagus which has been dealt with earlier) as target organ for tumour induction by all classes of NOC tested in various mammalian species. The administration route most commonly used here is oral intake. It is immediately evident from inspection that indirect-acting NOC are rarely active as inducers of alimentary canal cancers in rats (apart from the oesophagus), which may be held to be true for all vertebrate or mammalian species. The reason for this may safely attributed to the absence of activating enzymes along the canal.

On the other hand, indirect-acting NOC including α-acyloxyalkyl nitrosamines are largely effective as inducers of alimentary canal cancers when administered orally. The reason is fairly obvious, being due to the spontaneous decomposition of the NOC along the various segments of the alimentary canal.

Changes in *p*H along the segments of the canal would not be without their effects upon accumulation of the released ultimate carcinogen within these various segments. In general, it may be

assumed that the segment within which the *p*H is most favourable towards the NOC decomposition would be the one where DNA attack and modification is most prevalent.

The last factor concerns the properties of the organ segment involved in tumourigenesis, viz. low repair capacity, high cell turnover rate, and low immune defense. The relatively large amounts of ultimate carcinogen released as a consequence of oral intake followed by direct decomposition would most likely make up for any deficiencies in the target organ with regard to low repair capacity or low immune defense, so that the carcinogenesis process is not hindered by these factors. It is well-known that the various segments of the alimentary canal are continuously subject to cell renewal, so that the DNA turnover factor is ensured to be favourable towards tumourigenesis.

5. Bladder carcinogenesis in mammals

One of the most remarkable organotropic effects exhibited by nitrosamines is the induction of urinary bladder cancers in mammals of various species by DRNA containing an *w*-oxidised *n*-butyl group. Table VI.6 summarises the information on the available data for the induction of urinary bladder tumours in mammals by DRNA containing an *w*-oxidised *n*-butyl group. For the case of dibutylnitrosamine, urinary bladder cancer occurs owing to the metabolic formation of an *w*-oxidised derivative. The three factors of the model are now applied to rationalise these findings.

Although the commonest administration route here is oral intake, other route like sc injection produce the same effect,

Table VI.6 Summarises Urinary bladder as the choice of target organ by denoting the target organ, the structural type of carcinogen, the total number tested, the number positive, and the percentage positive.

Organ	Structural type	No.tested	No.+ve	% +ve
URINARY BLADDER				
	1. SYMMETRICAL	30	3	10.00
	(a) Unsubstituted	14	1	7.14
	(b) *C*-Oxidised	7	2	28.57
	2. UNSYMMETRICAL	118	13	11.02
	(a) Unsubstituted	53	4	7.55
	(b) *C*-Oxidised	47	9	19.15
	B. N-nitrosourea	54	1	1.85

indicating a true case of organotropism. The concept of direct and indirect metabolism described above find the relevant application here. Following administration, the organ of primary metabolism is liver, where enzymatic *w*-oxidation of an *n*-butyl group occurs for substrates like dibutylnitrosamine which have no *w*-oxidised moiety already present. For those NOC already possessing an *w*-oxidised butyl group, primary metabolism in the liver may not be necessary according to this model.

The *w*-oxidised form of the nitrosamines gets transported as a glucorunide or as ester to the urinary bladder in the process of excretion. The model assumes the existence in the bladder of *α*-hydroxylase enzymes specific for these *w*-oxidised substrates. thus the organ of primary metabolism is not the same as the organ for *α*-hydroxylation (the bladder) for dibutylnitrosamines. Substrates which are already *w*-oxidised at the time of administration not need primary metabolism in the liver but are transported straight to bladder in the form of soluble esters.

The bladder is the organ of critical DNA modification and tumourigenesis. As such, it should be able to promote the persistence of the alkylated lesions through ineffective repair capacity and also have an appreciable cell turnover rate. However, since accumulation of the *w*-oxidised carcinogen in the bladder is quite substantial, the high level of concentration of the carcinogen might be able to supplement for any deficiency in the target organ.

6. Pancreatic tumours in Syrian hamsters

β-oxidised *n*-propylnitrosamines are noteworthy for inducing pancreatic tumours in Syrian hamsters and no other species.

Table VI.1 summarises the available information for the induction of pancreatic tumours in Syrian hamsters by β-oxidised *n*-propyl-nitrosamines. This is a genuine organotropic effect. Oxidation at both β-positions of two *n*-propyl groups is apparently a necessary factors.

The mode of administration commonly employed here is an *sc* injection. The carcinogen may then get distributed through the circulatory system via the capillaries of the subcutis to eventually reach the pancreas.

The pancreas is the organ where, according to this model, α-hydroxylation occurs. The model assumes the existence of enzymes in the pancreas which are specific for substrates that get β-oxidised on both the *n*-propyl groups, not just one. α-hydroxylation at any of this group would release the necessary ultimate carcinogen.

The pancreas being the organ of tumourigenesis, it would also be the organ where critical DNA modification occurs. As such, the characteristic of low DNA repair capacity, appreciably cell turnover rate and low immune defense profile would be expected to be present.

7. Local-acting tumours in various species

Only direct-acting NOC are capable of inducing local tumours around the site of administration. The route commonly employed here are subcutaneous injection (resulting in subcutis tumours), topical application (resulting in skin tumours), and oral intake (resulting in alimentary canal tumours). Alimentary canal tumour following oral intake have already been dealt with earlier in this section.

The relative instability of the direct-acting parent carcinogen ensures its facile decomposition in and around the site of administration. Decomposition enables direct release of the *uc* in the affected area, where no metabolising enzyme is required. This model thus, eliminate the need for α-hydroxylating enzymes in the affected organs, viz. the skin and subcutis. Only those NOC possessing sufficient *in vivo* stability would be able to affect the organs or systems far remote from the site of administration, as is typified by the neurotropic activity of nitrosoureas.

The target organ (skin or subcutis) is thus seen to be the site of administration, decomposition, release of ultimate carcinogen, attack and modification of DNA, and tumourigenesis as well. As such, the target organ would be expected to possess the ability to retain the alkylated DNA lesions, ensured cell division and multiplication, as well as of low immune defense against the cancer cells formed. Here again the high level of concentration of the carcinogen administered might be able to overcome any setback in the target organ.

VI.5 *Concluding Remark*

Although the exact mechanism of the complexed organ-specificity of chemical carcinogenesis is not well established as yet, it is convincing to state that the above described mechanistic models should bear a commenable findings to the phenomenon of organ-specificity and target organ choice. The remarkable organ-specificity and the choice of target organ in test animals of different species and strains (mammals) displayed by the diferrent class of NOC is quite noteworthy. This characteristic

feature of organotropism and the choice of target organ demarcate a clear distinction between the direct and indirect-acting carcinogen in their induction of tumours in various organs.

The route of administration, the trasport site, and the *in vivo* stability of the carcinogen play important factors in organ-specificity of tumour induction and the choice of target organ for direct-acting carcinogen. It may not be so for the indirect-acting carcinogens. However, the presence and distribution of the metabolising enzymes in various target organ, followed by the nature of oxidation pathways whether direct or indirect mode of metabolism contribute a major deciding factors for indirect-acting carcinogens. In this connection, the presence of the metabolising enzymes being able to distinguish the structural differences like symmetrical or asymmetrical dialkylnitrosamines or even cyclic nitrosamines within the respective organ would also be a decision making criteria for organ-specificity. The effects of *pH* favourable for the biotransformation of direct-acting carcinogen through spontaneous decomposition *in vivo* to yield the reactive species would carry a more significant weightage rather than the presence of metabolising enzymes in the organ.

Apart from the above factors like the administration route, the organ of metabolism and their *in vivo* stability, the other relevant factors involved in the choice of target organ and its organ-specific tumour induction include the type and extent of critical DNA modification, the repair capacity, and the DNA cell turnover rate. To this the persistence of O^6-alkylguanine and O^4-alkylthymines in a particular organ correlates well with the

tumour susceptibility of the organ. Nevertheless, the presence and distribution of the DNA repair enzymes in respective organ is undoubtedly an important factors for organ susceptibility. Exposure to tissue specific tumour promotors may be another fatal criteria for organ-specificity by NOC.

It may be noted here that cell division, growth and maturity is a carefully regulated process in response to specific needs of the body. The balance between cell loss and cell renewal is steadily maintained in most organs during cell differentiation, amplification and maturation of stem cells. But the progenitors of the malignant cells in carcinogenesis is unabled to achieve full maturity. Here the continuous proliferations of immature malignant cells exceeds the number of cell loss. Thus the DNA cell turnover rate of immatured unwanted malignant cells become much higher than the rate of cell loss resulting to grow in a clone of cells, ultimately leading to formation of tumour.

This newly framed mechanistic models thus carry its own validity being based on the current evidence of data gathered from both experimental and theoretical findings. The application of the models to these various target organs seem to work well in explaining and predicting the choice of target organ and their organotropic effects so far applied here. Hepatocarcinogenesis by symmetrical dialkylnitrosamines is assigned as an outcome of direct metabolism, while the oesophagal tumours asymmetrical nitrosamines, pancreatic tumours β-oxidised n-propyl, and urinary bladder tumours by w-oxidised n-butyl are noted as indirect metabolism effects towards cancer induction.

To a certain extent, the application of this mechanistic models successfully help explain/predict and interpret the choice of target organ selectivity and their organotropism. Nevertheless, much research work has to be done to fully understand the exact mechanism of organ-specificity and the choice of target organ by *N*-nitroso compounds.

CHAPTER SEVEN.

SUGGESTIONS AND CONCLUSION.

CONTENTS:

VII.I. Fulfilment in Aims and Objectives of the Thesis.

In fulfilment of the aims and objectives of this research thesis is accomplished quite substantially and with great satisfaction. The handling of the research problems includes the extensive survey of the work, formulations of the research problems, its dimensional approach to solve the formulated research problems, and finally solving the formulated research problems itself and its logical interpretation of the output results. The chief objectives of the present research investigation has been formulated and described in chapter one. In an attempt of the work done towards the fulfilment of this research aims and objectives, following is the work accomplished.

The first aim and objective is to evolve the carcinogenic activity and inactivity of N-nitroso compounds. This objective has been tackled successfully well in two parts, in which chapter one and two serve as the key note to approach the formulated problems.

The phenomenological feature of the carcinogenic activity and inactivity of N-nitroso compound was solved in part by framing a series of mechanistic criterions employing the Ultimate Carcino-

gen Theory as the pivotal concept, described in chapter three. Using this mechanistic models, a thorough examination of the presence or absence of α-hydrogens, its reactivity or susceptibility and the role of UC hydrolytic decomposition in vivo, steering along the main activation pathways produce convincingly a favourable result in assigning the criteria. This positive result has been commendably supported by a series of sample calculations using *AM_1 MOPAC* package and scheming a series of cyclisation reaction mechanism yielding some inactive products.

The crucial role of modified DNA in the determination of the presence or absence of carcinogenicity is successfully handled, using Somatic Mutation Theory as its key concept, which is discussed in chapter four. Basing upon this conceptual mechanism, the proposed criterions of O-selectivity of DNA modification, the role of Watson-Crick protons acidity, and the conformational role of the exocyclic O-alkyl group on O-alkylated DNA bases are satisfactorily substantiated. Theoretical calculations using the AM_1 semi-empirical molecular orbital method serve to furnish some support for the reasoning behind this model.

The net result of the above two complete set of criterions output yield a good demarcation between the active and inactive members is achieved among the class of some 332 N-ntroso compounds so far tested alongwith the predictions of some untested compounds.

The second objective evolves the study of the well-observed variations of carcinogenic potency among the class of active N-nitroso compounds. The investigation has been possible to carry

out convincingly by employing the dose-response data of Druckrey *et al.*, coupled with the quantification scheme of Wishnok and Archer. It is observed that the value of our theoretically derived potency index (K_c) is closely consistent with the experimental carcinogenic potency index (I_c). This consistency is satisfactorily observed for hepatocarcinogenesis by different groups of dialkylnitrosamines such as *AA* type, *BB* type, *CC* type, and others as per *ABC* classification scheme. Although, there seems to be certain discrepancy between the theoretically derived potency index with that of experimental potency index, the output results is fairly convincing and satisfactory, which is being described in chapter five.

The third research objective put forward the application of mechanistic and physiological criteria to explain the choice of target organ and the remarkable organ-specificity of N-nitroso compounds. The organ-wise carcinogenic effects of NOC is confined to some 8 different organ systems such as the carcinogenicity of liver, oesophagal, respiratory tract, alimentary canal, nervous system, urinary bladder, pancreas, and skin. To all these organs carcinogenicity, a mechanistic and physiological criterions are applied for rationalisation and prediction, as to why different class or sub-class of NOC exhibit a distinct organ-specificity. Some of the clear example of this type include the symmetrical DRNA specific for liver cancer, asymmetrical DRNA specific for oesophagal cancer, and N-nitrosoureas specific for nervous system cancer. They all work well within the frame work of this mechanistic and physiological models for predictions and rationa-

lisation

VII.2 *Some suggestions for further studies*

Notwithstanding, within and without the content of this thesis, a number of research work is still expected to improve the phenomenon of carcinogenesis by N-nitroso compounds. A thoughtful criticism and suggestions to the present thesis work are also expected and welcome for better improvement. Out of which research field on N-nitroso carcinogenesis may open up a wider avenue for better refinement of the field. Following are the a few suggestions for further investigations.

(a) *Organ-wise distribution of oxidising enzymes*

The work described in this thesis concerning the presence and distribution of oxidising enzymes is limited to the current availability of experimental data of a few biological system published in various scientific journals. As for example, the presence of oxidising enzymes has not been reported in nervous system, which may be well related to non-carcinogenic effects of the indirect-acting N-nitrosamines. In this manner, the specificity of smmetrical DRNA for liver carcinogenesis, asymmetrical DRNA for oesophagal and cyclic nitrosamines for respiratory tract could well be established. This may be possible only after knowing the presence of the structure-dependent enzyme-specific, the nature of the fit between this enzyme and the carcinogens, and the type of oxidation pathways within the respective organ.

(b) *Quantification of carcinogenic potency index*

Apart from the experimental and theoretical carcinogenic potency index derivation of liver and oesophagal carcinogenesis

by dialkylnitrosamines, quantification could also be extented to some other organs like nervous system, respiratory tract, urinary bladder, mammary gland etc. This in turn would be related to the degree of carcinogenicity of each class of N-nitroso compound and its organ susceptibility.

(c) *Organ-wise distribution of DNA repair enzymes*

A more rigorous works are yet to be done concerning organ-wise distribution of DNA repair ezymes and its repair capacity. For many of the organs like in nervous system repair activity pertaining to O^4-alkylated or even O^6-alkylated have not been fully studied. For the organ-specificity of NOC, the role of DNA repair capacity would be important criteria. This will include the screening of the presence or absence of DNA repair enzymes, th ir exact mechanism of repair and the repair capacity in a partcular organ. Which in a way would manifest the carcinogenic activity of N-nitroso compounds, following the role played by the repair enzyme efficiency in the context of activation and detoxification pathays. Further research avenue is therefore awaited to explore in greater depth in the field of carcinogenesis by N-nitroso compounds, which may be directed to the study of organ-specificity and the role involved by DNA repair enzyme in the respective organ.

(d) *DNA cell turnover rate*

Cell turnover rate which is suppositely one of the factors involved in the organ-specificity is a less clear phenomenon. This may be well observed and could be related to the rate of DNA replicating cells of various organs of varying cells multiplica-

tions rate and of having finite life span of the progenitors. If the occurence of malignant cells are predominant in cells maturation arrest, then the exact mechanism of the maturation arrest of tumour cells is less clear and known. As such a better verification and confirmation of the cell turnover rate due to maturation arrest may also be further promising research area to work on.

(e) *Mechanism of cyclisation reaction to inactive products.*

Some of the ultimate carcinogen species so formed in vivo are proned to undergo further cyclisation reaction to yield some inactive products which do not have any significant carcinogenic effects. The consequence of this product manifest itself as following a detoxification pathways. The nature of this cyclisation reaction mechanism may be approximated through theoretical investigation or by experimental determination.

VII.3 *Concluding Remarks*

In fulfilment of the salient aims and objectives of this research work, the onset concluding note expresses the inner feeling satisfaction of the work done. Inspite of the limitations of facilities and working methods, the formulated research problem is being able to synthesize in a comprehensive manner. The extensive survey of the work done in N-nitroso carcinogenesis and conceptual approach, lend a valuable insight to the handling of this research objectives.

The current knowledge of the ultimate carcinogen theory contribute a frontal light to the solving of the parent

carcinogen stability *in vivo* and its biotransformation to reactive species towards the manifestation of carcinogenic activity and inactivity. While the concept triggered by the role of modified DNA and its oncogene products is an important contributing factors towards the induction of tumours. Thus the precise term of molecular event in the process of carcinogenesis have been possible to derive by this mechanistic models drawn from these two concepts. The prediction and rationalisation of the presence or absence of carinogenic activity among different class of NOC is reasonably satisfactory. The sample calculations on the O-selectivity of DNA alkylations, Watson-Crick protons acidity, and the role of the conformational barrier of O-alkylated DNA bases using AM_1MOPAC package demarcate a clear distinction among the active and inactive members of NOC to support this models.

Chapter on the N-nitroso carcinogenic potency studies in a couple of organs carry its own cridit of success, which in future may a useful reference of index for untested NOC and the organ system whose carcinogenic potency values have not been work out. This include the derivation of our numerical index for assigning the theoretical potency values in conjunction with the experimental potency index.

Appreciably, to a major extent, the result on organ specificity and the choice of target organ by N-nitroso compound is fairly a convincing accomplishment. Although a lot of experimental verification still awaits for organ specificity, nevertheless we have promising confidence that this mechanistic and physiological models would a medium of indicator to help solve the problems of

organ-specificity of N-nitroso compounds in broader spectrum. In true sense, more research avenue in the field of N-nitroso carcinogenesis may stem from the study of organ-specificity in test animals.

To pave way for better understanding and improved quality assimilation of the sequence-wise characteristic feature of carcinogenesis by N-nitroso compound, a couple of suggestions and directions for further studies have been proposed. Which undoubtedly may strengthen the mechanistic models worked out in this thesis. Finally, the use of more accurate methodology either in experimental or theoretical studies in the field of chemical carcinogensis should help solve the unsolved complex multi-stages process of cancers, especially with relevant to human cancers.

LIST OF ABBREVIATIONS

A = adenine

AAT = alkylguanine-DNA-alkyltransferase enzyme

Ae = 2-acetoxyethyl

AM_1 = Armsterdam Model

Bu = *n*-butyl

C = cytosine

C-*onc* = cellular oncogene

Cy = cyanomethyl

DNA = deoxyribose nucleic acid

DRNA = dialkylnitrosamine

Et = ethyl

G = guanine

G^* = guanine tautomer

GIT = gastrointestinal tract

He = 2-hydroxyethyl

HOMO = highest occupied molecular orbital

I_c = experimental potency index

K_c = theoretically derived potency index

LUMO = lowest unoccupied molecular orbital

Me = methyl

MNDO = modified neglect of differential overlap

MO = molecular orbital

NA = nitrosamines

N^7-G = N^7-guanine

N^7-RG = deprotonated N^7-alkylguanine

N^7-RG^+ = cationic N^7-alkylguanine

NOC = N-nitroso compound

O^6-G = O^6-guanine

O^6-RG = deprotonated O^6-alkylguanine

O^6-RG^+ = cationic O^6-alkylguanine

O^4-T = O^4-thymine

O^4-RT = deprotonated O^4-alkylthymine

Pe = pentyl

Pr = *n*-propyl

Pr^i = isopropyl

R = alkyl group

R^+ = alkyl cation

RNN^+ = alkyldiazonium ion

RNNOH = alkyldiazohydroxide

RNU = alkylnitrosourea

SCF = self-consistent field

sec = secondary

T = thymine

tert = tertiary

V-*onc* = viral oncogene

1. RESEARCH PAPER ONE

TITLE :*THEORETICAL INVESTIGATIONS ON CHEMICAL CARCINOGENESIS BY N-NITROSO COMPOUNDS AND ALKYLATING AGENTS: A REVIEW*

AUTHORS :*James Haorah and R.H. Duncan Lyngdoh*

REF :*Ready for communication*

ABSTRACT: This review discusses the applications of theoretical and mechanistic methods to the problem of carcinogenesis by N-nitroso compounds and alkylating agents, basically focussing on molecular mechanisms of the phenomenon. Most of the theoretical methods deduced from the nucleophilic character of the sites on cellular macromolecules attacked by chemical carcinogens. Much experimental efforts has gone into the identification of putative proximate and ultimate carcinogen species for various classes of chemical carcinogens. A large body of theoretical work has also been done which takes the cue from ultimate carcinogen concept.

2.RESEARCH PAPER TWO

TITLE : *THE MECHANISTIC CRITERIA FOR PRESENCE OR ABSENCE OF CARCINOGENIC ACTIVITY IN N-NITROSO COMPOUNDS I. ROLE OF THE PARENT, PROXIMATE AND ULTIMATE CARCINOGEN SPECIES*

AUTHORS : *JAMES HAORAH & R.H. DUNCAN LYNGDOH*

REF : *Ready for communication*

ABSTRACT : The ultimate carcinogen hypothesis provides a vital key to current understanding on the molecular mechanisms underlying carcinogenic activity in chemicals. The concept of this hypothesis is applied in conjunction with the wealth of the existing data on the molecular mechanisms of N-nitroso carcinogenesis to frame series of criteria which could help decide whether a given N-nitroso compounds would be carcinogen or not. Such screening for carcinogenesis on purely mechanistic basis could possibly furnish a viable and interesting alternative to the expensive and laborious long-term screening tests using laboratory animals.

3. RESEARCH PAPER THREE

TITLE : *THE MECHANISTIC CRITERIA FOR THE PRESENCE OR ABSENCE OF CARCINOGENIC ACTIVITY/INACTIVITY IN N-NITROSO COMPOUNDS II. ROLE OF THE MODIFIED DNA*

AUTHORS : *JAMES HAORAH & R.H. DUNCAN LYNGDOH*

REF : *Ready for communication*

ABSTRACT : The concept of Somatic Mutation Theory delineate the sequence of molecular understanding of modified DNA and its onco-proteins products for chemical carcinogenesis. The mechanistic role of this modified DNA is applied here to assign the carcinogenic activity or inactivity of a given N-nitroso compound. A series of mechanistic criterions is thus adopted here for the conferring of mutagenic or non-mutagenic properties on to DNA, which take the form of sufficient O-selectivity of alkylation, sufficient acidity of the Watson-Crick protons of the alkylated bases, and the role of conformational barrier for exocyclic O-alkyl groups in O-alkylated bases.

4. RESEARCH PAPER FOUR

TITLE : *MECHANISTIC FACTORS MODULATING CARCINOGENIC POTENCY OF CARCINOGENIC DIALKYLNITROSAMINES*

AUTHORS : *JAMES HAORAH & R.H. DUNCAN LYNGDOH*

REF : *Ready for communication*

ABSTRACT : Carcinogenic N-nitroso compounds display a wide range of tumour-inducing power in rats, expressible in the form of a numerical index I_c of relative carcinogenic potency. Taking the cue from the wealth of information available concerning the carcinogenesis pathways for N-nitroso carcinogens, a series of mechanistic factors are proposed which could have bearing on carcinogenic potency. These factors are quantified employing various factors theoretical considerations and also using the semi-empirical AM_1 SCF molecular orbital method, their values then being compared with the values of the potecy index I_c. The patterns of correlations are posutive in many cases, and these are discussed with reference to the bearing they could have for the mechanism of carcinogensis by these compounds.

5. RESEARCH PAPER FIVE

TITLE : *TOWARDS THE QUANTIFICATION OF MECHANISTIC FACTORS MODULATING CARCINOGENIC POTENCY OF DIALKYLNITROSAMINES*

AUTHORS : *JAMES HAORAH & R.H. DUNCAN LYNGDOH*

REF : *Ready for communication*

ABSTRACT : Using postulated mechanistic pathways for dialkylnitrosamines carcinogenesis, a series of mechanistic factors of possible relevance for carcinogenic potency are identified and quantified, which include factors for α-hydroxylation feasibility, for hydrolytic loss of the ultimate carcinogen species, for O-selectivity of DNA alkylations, and for the role of the DNA repair enzymes. These factors are incorporated in the form of a single umerical index for parent carcinogenic potency, whose derivation is give here. Some of the mechanistic factors present in this index are quantified after appropriate semi-empirical comparison with the experimental carcinogenic potencies in a few instances, and the theoretical index thus constructed is used to furnish predictions of potency for other members of the class. The values of the mechanistic factors for O-selectivity reflect the trends expected from structure, from molecular orbital calculations and from experimental observations. Through this feed-back between theory and experiment, the dialkylitrosamines are assigned values for the theoretical potency index, furnishing an ordering which is thoroughly substantiated by experimental values for relative carcinogenic potency.

6. RESEARCH PAPER SIX

TITLE : *THE MECHANISTIC CRITERIA FOR THE CHOICE OF TARGET ORGANS AND ITS ORGANOTROPIC EFFECTS OF TEST ANIMALS IN N-NITROSO CARCINOGENESIS.*

AUTHORS : *JAMES HAORAH & R.H. DUNCAN LYNGDOH*

REF : *Ready for communication*

ABSTRACT : The remarkable organ-specificity of N-nitroso compound in test animals is well documented. The choice of target organ by N-nitroso compound play a key criteria for manifestation and assessment of the carcinogenic effects in test animals. Heuristic factors such as the route of administration, the distribution of metabolising enzymes, the role of modified DNA repair capacity, and the cell turnover rate seem to govern the phenomenon of organ selection and its organ-specificity of animals by N-nitroso carcinogenesis. The application of this mechanistic model provides a viable logistic results.

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