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Carcinogenicity of nitroaromatics

Bc thesis

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I certify that I worked out this Bc thesis self-containedly under the supervision of Doc. RNDr. Marie Stiborová, DrSc. and I certify that I have properly cited all used sources.

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CONTENT

LIST OF ABBREVIATIONS 5

AIMS OF THE THESIS 7

1. CANCER AND CARCINOGENESIS 8

1.1 Cell division mechanism 8

1.2 Carcinogenesis 9

1.3 Mechanism of carcinogenesis 9

1.4 Factors of carcinogenesis 12

1.5 Chemical carcinogens 15

2. BIOTRANSFORMATION OF CARCINOGENS 15

2.1 Phase I biotransformation 16

2.2 Phase II biotransformation 17

2.3 Mixed-function oxidase system (microsomal monooxygenase system) 19

2.3.1 Cytochrome P450 19

2.3.2 Mono-oxygenation catalytic cycle 22

2.4 Enzymes metabolizing the xenobiotics by reduction mechanism 24

2.4.1 DT-diaphorase 24

2.4.2 Xanthine oxidase 25

3. NITROAROMATICS 26

3.1 Parent compounds of nitroaromatics 26

3.1.1 Benzanthrone 26

3.1.2 Polycyclic aromatic hydrocarbons 27

3.2 Metabolism of nitroaromatics 28

3.3 Nitroaromatics and human health 29

3.3.1 Nitrated polycyclic hydrocarbons 30

3.3.2 3-Nitrobenzanthrone 31

3.3.3 2-Nitrobenzanthrone 34

3.3.4 2-Nitroanisole 34
3.3.5 Aristolochic acid 37
3.3.6 Nitropyrenes 39

CONCLUSION 42
REFERENCES 43

LIST OF ABBREVIATIONS

AA	aristolochic acid
AAI, II	aristolochic acid I, aristolochic acid II
AAN	aristolochic acid nepropathy
3-ABA	3-aminobenzanthrone
BaP	benzo[a]pyrene
CHN	Chinese herbs nepropathy
CYP	cytochrome P450
CYP1, 2, 3, 4	cytochrome P450 families
COX	cyclooxygenase
DNA	deoxyribonucleic acid
DS DNA	double-stranded deoxyribonucleic acid
DNPs	dinitropyrenes
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide (quinone form)
$FADH_2$	reduced flavin adenine dinucleotide (hydroquinone form)
FMN	flavin mononucleotide
GI-tract	gastrointestinal tract
IARC	International Institute for Research on Cancer
LPO	lactoperoxidase
MFO	mixed-function oxidase system
MPO	myeloperoxidase
2-NA	2-nitroanisole
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$	oxidised nicotinamide adenine dinucleotide phosphate
NATs	*N,O*-acetyltransferases
2-NBA	2-nitrobenzanthrone
3-NBA	3-nitrobenzanthrone
N-OH-ABA	*N*-hydroxy-3-aminobenzanthrone

Nitro-PAH	nitrated polycyclic aromatic hydrocarbons
NPs	nitropyrenes
1-NP	1-nitropyrene
2-NP, 4-NP	2-nitropyrene, 4-nitropyrene
NQO1	NAD(P)H:quinine oxidoreductase
PAH	polycyclic aromatic hydrocarbons
PAPS	phosphoadenosine phosphosulphate
ROS	reactive oxygen species
SULTs	sulphotransferases
UDP	uridine 5'-diphosphate
XO	xanthine oxidase

AIMS OF THE THESIS

Environmental pollution is becoming an important public health problem, due to increasing worldwide urbanization and increasing consumption of fossil fuels. The aims of this thesis are to present the background of an important group of environmental pollutant, nitroaromatics, and to summarise facts of their occurrence and metabolic activation.

The attention is mainly focused on 3-nitrobenzanthrone and 2-nitroanisole, strong carcinogens and mutagens for rats and potential carcinogens for humans.

1. CANCER AND CARCINOGENESIS

Cancer is a disease spread worldwide. Nowadays, this civilization illness is one of the leading causes of death along with cardiovascular diseases. The occurrence of cancer is still increasing and it affects younger age groups [1]. It is known that the major causes of cancer are lifestyle factors like nutrition habits, stress, sun exposure or toxic and carcinogenic contaminants of the environment. Not only environmental factors but also individual genetic susceptibility plays an important role in many human cancers.

Lung cancer is the most common malignant disease worldwide and is the major cause of death from cancer [2]. Although tobacco smoking is the overwhelming cause of lung cancer, vehicular exhaust and ambient air pollution are also implicated as contributory factors.

1.1 Cell division mechanisms

Cancer is now defined as a group of diseases that are characterised by defects in signal transduction causing uncontrolled, abnormal growth of cells and loss of differentiation [3].

This causes cancer cells different from healthy cells. In healthy organism, the cells normally remain under strict development control and the gene expression is regulated continuously, which keeps the tissue cells under the differential condition. Various types of proteins secure the gene expression regulation (e.g. receptors, protein kinases, transcriptional factors, growth factors). Genes responsible for these proteins formation are assigned as *proto-oncogenes*, and they are potentially capable of transforming into *oncogenes*. Mainly the oncogenes products that are released after the proto-oncogenes activation, e.g. the oxidative changes to DNA caused by the forms of oxygen radicals, affect the tumour growth [4].

Besides proto-oncogenes a completely different group of genes, which are assigned as *anti-oncogenes* (tumour suppresser genes), participate in the cells development control by inhibiting cell proliferation. Normally the balance between proliferation and programmed cell death is maintained by regulating both processes to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer disrupt these processes by interrupting the regulating processes [5].

1.2 Carcinogenesis

Carcinogenesis (often used synonymously with transformation, tumourigenesis) is a general term to denote the process of development of neoplasm [6].

Tumour (neoplasm) is characterised as an abnormal mass of tissue that results from excessive cell division that is uncontrolled, progressive and performs no useful body function. By the ability of cancer cells to spread through the bloodstream and lymphatic system to other parts of the body, tumours can be assorted as:

1. **Benign tumours**, not cancerous, grow by simple expansion and often remain encapsulated by a layer of connective tissue. Benign tumours are rarely life threatening, although if they occur in an enclosed space such as in the brain or secrete large amounts of certain hormones, they can be lethal [7].
2. **Malignant tumours** (cancers) grow in an invasive manner and shed cells that, in a process known as **metastasis**, colonize new sites in the body. Malignant tumours are almost invariably life threatening [7].

1.3 Mechanism of carcinogenesis

Carcinogenesis is a multi-step process involving DNA damage. The key damage, responsible for development of cancer cells, occurs in genes regulating normal cell growth and division. The development of cancer can be divided into three stages: initiation, promotion and progression followed by malignant conversion [3].

The scheme of the carcinogenesis process is shown in **figure 1**.

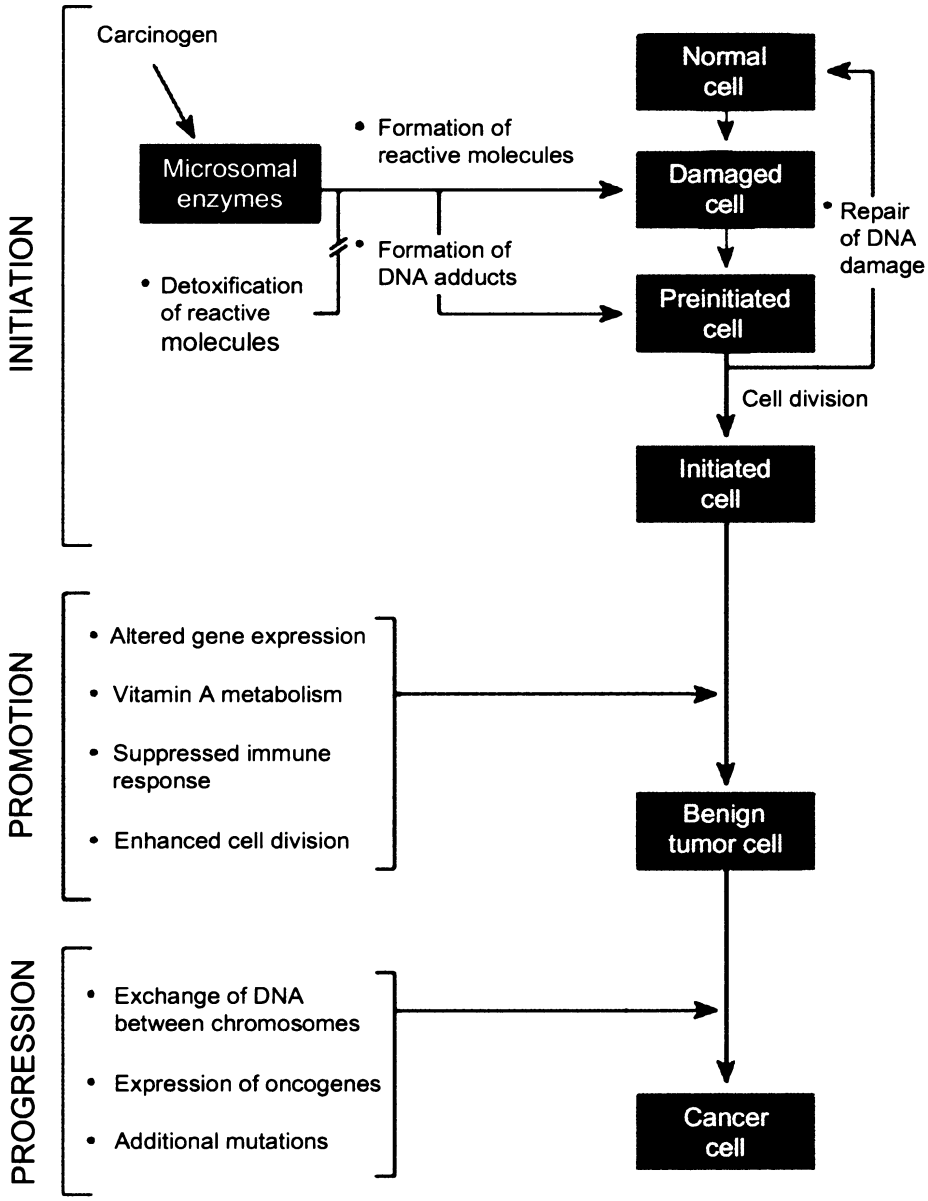


Figure 1: Process of carcinogenesis [8].

The **initiation** stage reflects a permanent and irreversible change in the initiated, originally healthy, cell [6]. Initiation results from exposure of cells to an appropriate dose of a carcinogenic agent (initiator). An initiated cell is in some manner altered, rendering it likely to give rise to a pre-carcinogenic lesion, subsequently to a tumour [9].

As mentioned, the newly formed pre-carcinogenic lesion is induced by several various modifications in the key genes of DNA and changed into reactive form (ultimate carcinogens).

DNA represents a unique target for carcinogenic agents and for treatment of various types of diseases as well. Carcinogens (and drugs) can target the nucleotide sequences present in DNA and disrupt their normal cellular function [10].

Although most of the modifications are eliminated from DNA by repair mechanisms, some of the alterations endure. Un-repaired alterations in the DNA are essential first steps in the process of initiation [4]. Initiation alone, however, is not sufficient for tumour formation. For the change, in DNA, to be heritable, the damaged DNA template must undergo at least one cycle of proliferation, so that the change in DNA becomes fixed or permanent.

The second stage of carcinogenesis is **promotion**. This stage of carcinogenic process can last years to decades. During promotion, the initiated cell is stimulated by promoters to grow and divide faster and becomes a population of cells. Eventually, during this process a benign tumour becomes evident [3]. The factors that are assigned as promoters can induce initiated cells. Such compounds are non-tumorigenic by themselves. Furthermore, the effects of promoters are reversible and do not affect DNA directly [9]. Most of the promoters have the epigenetic effect, several chemicals belong to promoters.

In contrast to the stage of initiation, promotion may be continually modulated by variety of environmental factors, including frequency with which the promoting agent is administrated [6].

During the third stage of carcinogenesis, the **progression phase**, there is further growth and expansion of the tumour cells over normal cells. The genetic material of the tumour is more fragile and prone to additional mutations [3]. Agents that act during progression are assigned as progressors [11]. Their effect is similar to that of chemical initiators (carcinogens). They are capable of converting an initiated cell or a cell in the stage of promotion to a potentially malignant cell [6].

The DNA mutations in this stage occur in genes that regulate growth and cell function such as oncogenes, tumour suppressor genes, and DNA mismatch-repair genes. These changes significantly contribute to tumour growth until conversion occurs, when the growing tumour becomes malignant and possibly metastatic [3].

1.4 Factors of carcinogenesis

A large number of agents, extrinsic carcinogenic factors, cause genetic damage mentioned above and induce neoplastic transformation of cells [9]. Particularly by production of covalent adducts, hydroxyderivatives of the DNA bases, production of cyclic adducts and pyrimidin dimers, apurination and apyrimidination.

They fall into the following categories:

(1) Chemical carcinogens (e.g. the polycyclic aromatic hydrocarbons, aromatic amines)

(2) Radiant energy (e.g. ultraviolet rays, ionizing radiation)

(3) Oncogenic viruses (e.g. papillomaviruses, Epstein-Baar virus, hepatitis B virus)

Radiant energy and several chemical carcinogens are documented causes of cancer in humans, and the evidence linking certain viruses to human cancers grows ever stronger [9].

1.5 Chemical carcinogens

Chemical carcinogens include a great variety of man-made and naturally occurring substances [7]. They can be classified into three groups, in accordance with their impact on the molecules of DNA (**figure 2**):

- Genotoxic carcinogens cause irreversible genetic damage or mutations by binding to DNA covalently; they form covalent adducts [4].
- Carcinogens causing modifications in the DNA molecule's structure by various mechanisms like single- and double-strand breaks, inter- and intra-strand cross-linking (DNA-DNA cross-linking or DNA-protein cross-linking) [4].
- Epigenetic carcinogens modify the molecules of DNA noncovalently; they have an ability to intercalate into the DNA's double helix [12].

The production of the DNA adducts is regarded as the most serious one because more than 90% of established chemical carcinogens initiates the tumorous process by binding covalently to the DNA. The modification can persist for years, without any operative consequences or it can be repaired by the mechanism of reparations (cellular enzymes) [11].

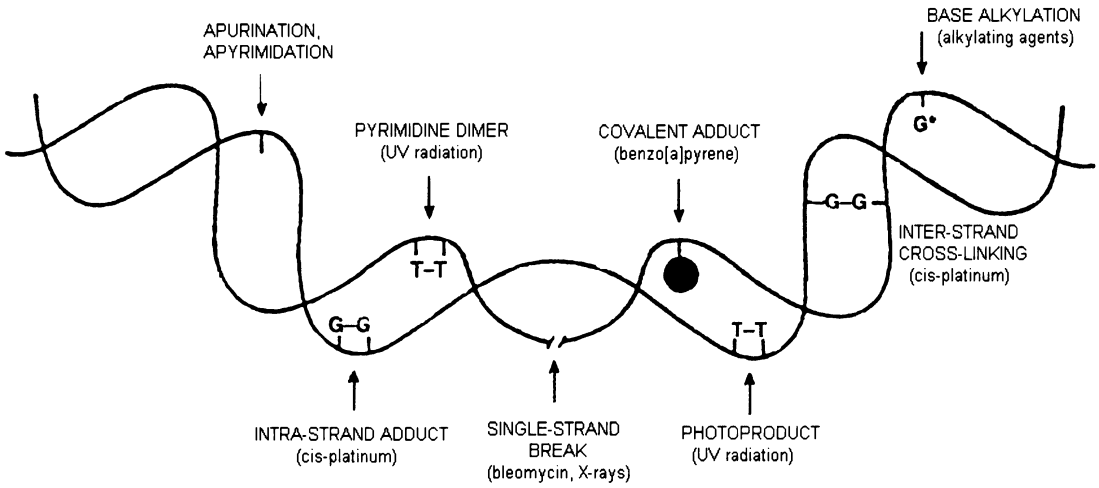


Figure 2: The mechanisms of DNA damage by various extrinsic factors [4].

It should be noted that some chemicals possess the capability of both initiation and promotion, as evidence of their ability to induce tumours without any added factors. They are called "complete carcinogens" to distinguish them from "incomplete carcinogens", which are defined as agents capable of only initiation [9].

Chemicals that initiate carcinogenesis are extremely diverse in structure and include both natural and synthetic products. They fall into one of two categories (**Table 1**):

(1) *direct-acting* compounds, which do not require chemical transformation for their carcinogenicity

(2) *indirect-acting* or *procarcinogens*, which require metabolic conversion *in vivo* to produce **ultimate carcinogens** capable of transforming cells [9].

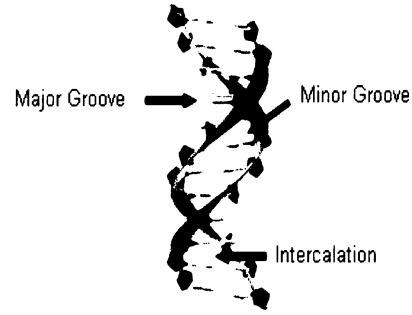
Table 1: Major chemical carcinogens (adapted from [9])

DIRECT-ACTING CARCINOGENS	PROCARCINOGENS THAT REQUIRE METABOLIC ACTIVATION
Alkylating agents	**Polycyclic and Heterocyclic Aromatic Hydrocarbons**
Beta-propiolactone Dimethyl sulphate Diepoxybutane Anticancer drugs (cyclophosphamide, chlorambucil, nitrosoureas and others)	Benz(a)anthracene Benz(a)pyrene Dibenz(a,h)anthracene 3-Methylcholanthrene 7,12-Dimethylbenz(a)anthracene
Acylating agents	**Aromatic Amines, Amides, Azo Dyes**
1-Acetyl-Imidazole Dimethylcarbomyl chloride	2-Napthylaminofluorene Benzidine 2-Acetylaminofluorene Dimethylaminoazobenzene (butter yellow)
	Natural Plant and Microbial Products
	Aflatoxin B Griseofulvin Cycasin Safrole Betel nuts
	Others
	Nitrosamine and amides Vinyl chloride, nickel, chromium Insecticides, fungicides Polychlorinated biphenyls

All direct-acting and ultimate carcinogens have one property in common: They are highly reactive electrophiles (have electron-deficient atoms) that can react with nucleophilic (electron-rich) sites in the cell. These reactions are mono-enzymatic and, as mentioned before, they result in the formation of covalent adducts, where DNA is the primary target [9].

Both major and minor grooves formations in DNA right-handed twist are lined by potential hydrogen bond donors and acceptors (**Figure 3**). So as a result, a variety of flat, polycyclic aromatic molecules are able to slip sideways, or intercalate between the stacked bases. Many cancer–causing and cancer-preventing agents function by interacting with DNA in this way. Both those agents can target the nucleotide sequences present in DNA and disrupt their normal cellular function. The cross-links formed by minor groove binders are less susceptible to repair by intracellular enzymes and results in the formation of stable adducts [10].

Figure 3: The cancer-causing/preventing agent's interaction sites in the DS DNA molecule [10].



Metabolic conversion, biotransformation, of carcinogens is described in chapter 2.

2. BIOTRANSFORMATION OF CARCINOGENS

Carcinogens are often compounds exogenous for individual organisms (xenobiotics).

Xenobiotics (xenos – adventitious, foreign) are defined as chemicals found in organisms, but not expected to be produced or present in them; or they are chemicals found in organism in much higher concentrations than usual [13].

The ways of xenobiotics transformation and disposal from the organisms are pursued by the mechanism called **biotransformation** [14].

The biological result of the interaction of the foreign compound with the biotransformation enzyme system depends on a variety of factors and can be predicted. Generally, biotransformation increases polarity and water solubility [15], and lipophilic xenobiotics that are metabolically stable, are made to be more hydrophilic, which results in facilitation of excretion of chemicals by the kidney (urine) and/or by faeces [5].

To increase hydrophilicity, a polar group is added or unmasked. In some cases, the added groups convert the xenobiotics into a weak acid or base that is predominantly ionized at physiologic pH [5].

According to the chemical structure of the xenobiotics and the enzyme system which transforms xenobiotics in the organism, **metabolic bioactivation** of the xenobiotics can occur instead of **metabolic inactivation**. A range of drugs, assigned as pro-drugs, needs that metabolic activation, but also 98% of genotoxic carcinogens are activated by that way (so-called "proximate" carcinogens). Only few of them are carcinogenic immediately (so-called "ultimate" carcinogens) [1].

Enzymes that are participating in the biotransformation of xenobiotics are present in the highest concentration in the liver, but are also located in organs that are linked to major routes of exposure (e.g. skin, lung, GI-tract) and in tissues like kidney, pancreas or placenta [16] (adapted from [65] [66] [67])

The process of biotransformation of xenobiotics is in animal kingdom divided into two phases – Phase I and Phase II reactions showed in **Figure 4**. Xenobiotics are first activated by hepatic enzymes (oxidation, reduction, hydrolysis and/or hydration of the xenobiotics), giving rise to the active secondary metabolite which is then conjugated with glucuronic or sulphuric acid, or glutathione, followed by excretion in bile or urine [17].

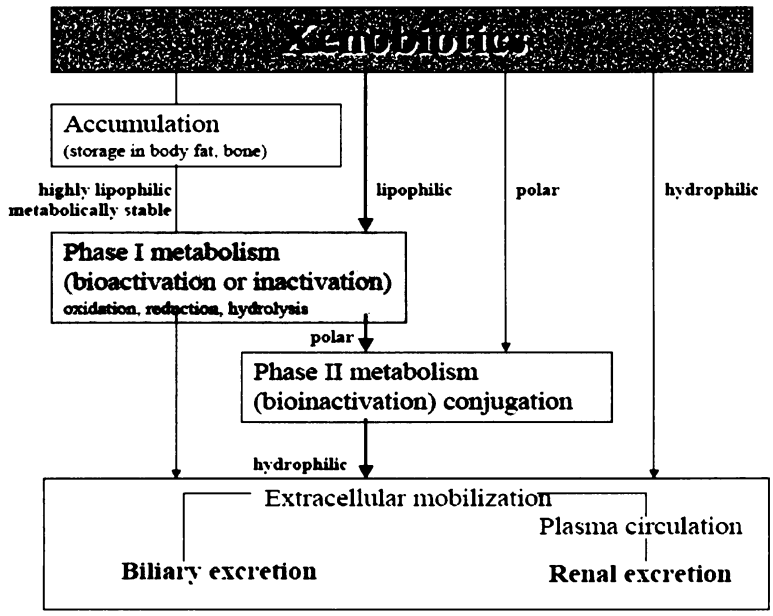


Figure 4: The process of biotransformation of xenobiotics [18].

2.1 Phase I biotransformation

Phase I reactions include introduction of such functional groups as –OH, -NH_2, -SH or –COOH, to the molecule of xenobiotics [16] (adapted from [68]) or functional groups already presenting in the molecule are unmasked [1]. Both these reactions, as mentioned, result in an increase in hydrophilicity of the compound.

Three types of reactions in this phase of biotransformation are distinguished:

- **oxidation** (e.g. C- and N-hydroxylation, dealkylation, deamination)
- **reduction** (nitro- and azo-reduction) – as an example of carcinogens activated by reduction is 2-nitroanisole, 1-nitropyrene, 1,6-nitropyrene, aristolochic acids
- **hydrolysis** (of esters and amides)

The majority of enzymes catalyzing these reactions are primarily located in the membranes of the endoplasmic reticulum, but they also occur in the cytoplasm, in the Golgi apparatus and in the extra cellular space. The key role in phase I biotransformation has a group of enzymes, which is termed as **Mixed-function oxidase system (MFO)** with **cytochrome P450** as a terminal oxidase [1]. The MFO catalyzes a wide variety of reactions including epoxidations, N-dealkylations, O-dealkylations, S-oxidations, and hydroxylations of aliphatic and aromatic substrates [5].
Other important enzyme systems that may participate in Phase I biotransformation of xenobiotics are: **NAD(P)H:quinone reductase (DT-diaphorase)**, **xanthine oxidase (XO)**, peroxidases and, under special conditions, aldehyde and alcohol-oxidases and dehydrogenases [12].

2.2 Phase II biotransformation

Phase II type of metabolism generally involves conjugation reactions. These conjugations help to make the compound even more polar by adding a large polar group to the compound by transferase enzymes, thus facilitating biliary and renal secretion [18]. However, in this conjugational phase xenobiotics can also be activated into even more toxic compound (e.g. conjugation with sulphate assisted by sulphotransferases) [14].

Phase II biotransformations include glucorionidation, sulphation, acetylation, and conjungations with glutathione and other amino acids (glycine, glutamine, cysteine, taurine and glutamic acid).

The enzymes responsible for these reactions are localised in the cytosol, with the exception of **UDP-glucuronosyltransferases**, which are the microsomal enzymes [16].

The UDP-glucuronyltransferases are strong detoxicants. They are localised within the smooth endoplasmic reticulum nearby the cytochrome P450 (Phase I enzymes). This intimate

relationship between cytochrome P450 and UDP-glucuronyltransferases facilitates "coupling" between certain Phase I and Phase II reactions [18].

In the conjugation phase, xenobiotics and endogenous compounds are also sulphated by **sulphotransferases** (SULTs), the enzymes responsible for these reactions. Sulphotransferases (a total of 11 different cytosolic SULTs have been detected by man), detoxicate xenobiotics but they can also activate the xenobiotics into even more toxic compounds. The conjugation product of sulphate with N-hydroxycompounds (resulted from the phase I of biotransformation) is not stable under the acid conditions of urine and decays to give rise to a nitrenium ion. Therefore, an electrophile is created which reacts with DNA and proteins. 3'-Phosphoadenosine-5'-phosphosulfate (PAPS) is the SULT's cofactor [14].

Acetylation is also an important Phase II pathway involved in the detoxification of a large number of chemical carcinogens and arylamines and hydrazine drugs. In the similar way as sulphotransferases, also **acetyltransferases** give rise to a nitrenium ion reacting with DNA and proteins [14].

Table 2 shows reactions and enzymes of Phase I and II of biotransformation of xenobiotics

Table 2: Comparing Phase I and Phase II reactions and enzymes (adapted from [19])

Enzymes	**Phase I**	**Phase II**
Types of reactions	Hydrolysis Oxidation Reduction	Conjugations
Increase in hydrophilicity	Small	Large
General mechanism	Exposes functional groups	Polar compound added to functional group
Consequences	May result in metabolic activation	Facilitates excretion

2.3 Mixed-function oxidase system (microsomal monooxygenase system)

The mixed-function oxidase system (MFO) is a multi-enzyme electron transport system, which plays a pivotal role in the metabolism of xenobiotics [20]. This system catalyzes a wide variety of reactions including oxidations, oxygenations, dealkylations and hydroxylations of xenobiotics, as mentioned above, using mainly molecular oxygen in the process [5]. The mixed-function oxidase system, which includes hydroxylase and one or two components enabling the electron transport, originates in the mitochondria [20] but it is predominantly localised in the membrane of the smooth endoplasmic reticulum [5].

It contains at least of three basic components [21]:

- **NADPH:cytochrome P450 reductase** – it is a flavoprotein enzyme, which functions as an electron pair divider gradually providing these electrons to cytochrome P450 [21].
- **Cytochrome P450** (CYP) – it is a hemethiolate enzyme containing protoporphyrine. It is a terminal oxidase enzyme of the MFO system [14].
- **Membrane lipids** – they cause the alterations in cytochrome P450 conformation, that increase its substrate specificity and they also stimulate the active complex cytochrome P450-NADPH:P450 reductase formation [1].

Facultatively, the MFO can also contain cytochrome b_5 and NADPH:cytochrome b_5 reductase [21].

2.3.1 CYTOCHROME P450

The cytochrome P450 (CYP) superfamily of constitutive and inducible enzymes is responsible for the catalytic hydroxylation of a multitude of compounds with diverse chemical structures and mode of action linked only by their lipophilic nature [22]. The cytochrome P450 enzymes are primarily located in the liver, but there is also substantial evidence that individual CYP enzymes are also expressed in other tissues [23].

Currently, 57 human CYP genes and 29 pseudogenes (nonfunctional) have been identified [24]. Many of these are used in the metabolism of sterols and vitamins A and D. About one quarter of the 57 CYPs are generally considered to be involved primarily in the metabolism of

xenobiotics [11]. These xenobiotic–metabolizing CYP enzymes comprise CYP1, CYP2, and CYP3 with some involvement from CYP4 families [23].

The xenobiotic–metabolizing CYP enzymes play a central role in the oxidative metabolism of a wide variety of endogenous and exogenous compounds including environmental carcinogens, plant toxins, and anti-cancer drugs. In addition, current developments in CYP-directed gene therapy (a prodrug activated by exogenous CYPs) are examined [23]. The CYP enzymes are probably involved in more bio-activation processes than other enzymes because they collectively have more substrates than any other enzymes [24]. They are also capable of deactivating anti-cancer drugs [23].

Microbial CYP enzymes play a key role in the bio-degradation of a wide variety of contaminants, such as pesticides, polycyclic aromatic hydrocarbons, nitroaromatics, etc [25].

NADPH:cytochrome P450 reductase is the "yellow protein" electron donor for several oxygenase enzymes found on the endoplasmic reticulum (microsomes) of most eukaryotic cells [26] and it catalyzes electron transfer from NADPH to all known forms of CYPs.

As mentioned before, NADPH:cytochome P450 reductase is a flavoprotein consisting of two function domains:

- N-terminal domain (hydrophobic), that works as a membrane anchor [21]
- C-terminal domain (hydrophilic) that contains both FAD- and FMN-bonding domains in equal proportions [21]

A series of studies established (based on the redox midpoint) that FAD is the electron acceptor flavin from NADPH and that FMN is the electron donor to acceptor proteins such as the cytochromes, as shown below in **Figure 5** [26]:

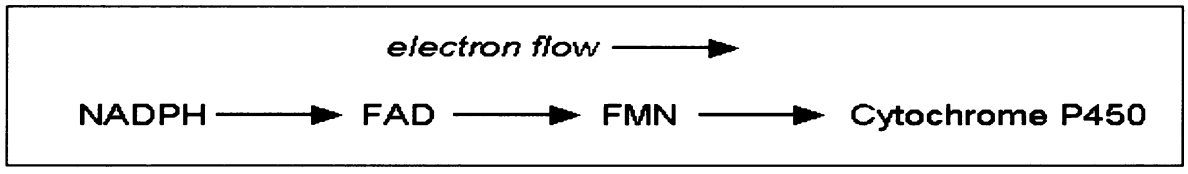


Figure 5: The role of flavins in electron flow [26].

Interactions between NADPH:cytochrome P450 reductase and CYP have primarily electrostatic character. The CYP's enzyme positively charged surface (lysine and arginine) interacts with the negatively charged surface of NADPH:cytochrome P450 reductase (**Figure 6**). Hydrophobic interactions of non-polar amino acids are applied at the area of membrane domains as well [21].

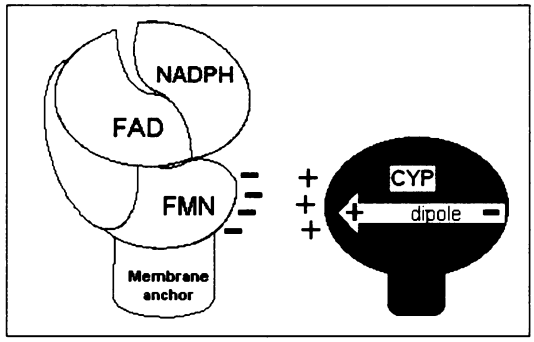


Figure 6: NADPH: cytochrome P450 reductase and CYP electrostatic interaction. The figure also shows the MFO components: NADPH: cytochrome P450 reductase, CYP and membrane lipids [26].

Beside the endogenous substrates (cytochrome P450, cytochrome c), NADPH:CYP reductase reductively metabolizes also low-molecular substrates and xenobiotics like nitro-aromatics e.g. 1,8-dinitropyrene, 3-nitrobenzanthrone, aristolochic acids. These exogenous substrates (xenobiotics) interact with the domain of the protein molecule of the enzyme, where the cofactor is bound to [21].

In addition, the cytochrome P450 enzymes are known to be active in the reduction of nitroaromatics. They metabolise 1-,2- and 4-nitropyrene, 6-nitrochrysene, 3-nitrobenzanthrone and aristolochic acids [27]. Those CYPs among these enzymes, that are known or suspected to be effective in the metabolism of polycyclic aromatic hydrocarbons (PAHs) or nitro-PAHs are:

CYP1A1: This enzyme catalyses oxidation of wide range of PAHs and nitro-PAHs. CYP1A1 is constitutively expressed in low levels in tissues [16] (adapted from [69]).

CYP1A2: It is a poor metaboliser of PAHs and nitro-PAHs, however it has been shown to be induced by benzo[a]pyrenes, a few benzofluoranthenes and benzoanthracenes [16] (adapted from [70]).

CYP1B1: This enzyme biotransforms a variety of PAHs and nitro-PAHs in most tissues [16] (adapted from [71]).

CYP2B6: Apart from PAHs, this enzyme can metabolise certain amino-compounds such as 6-aminochrysene [16] (adapted from [72]).

CYP2C8 and **2C9**: Apart from the metabolism of several endogenous substances, such as arachidonic acid and retinoic acid, **CYP2C8** can diminish the effect of the anti-cancer drug Paclitaxel. **CYP2C9** is involved in metabolising several non-steroidal anti-inflammatory drugs, as well as xenobiotics like warfarin [16] (adapted from [73,74]).

CYP2D6: This enzyme has a wide variety of substrates; however the enzyme itself appears to play little or no role in the activation of chemical carcinogens [16] (adapted from [68]).

CYP3A4: This enzyme is inducible by numerous substances and is known to transform steroids as well as xenobiotics such as benzo[a]pyrene, 6-aminochrysene, 1-nitropyrene, and aflatoxin B1 and G1[16] (adapted from [68]).

2.3.2 MONO-OXYGENATION CATALYTIC CYCLE

The basic reaction catalysed by CYPs is **mono-oxygenation** by incorporating one atom from an oxygen molecule (O_2) into a hydrophobic substrate and reducing the other oxygen atom to water with the reducing equivalents derived from NADPH [21]. The reaction cycle of cytochrome P450 enzyme consists of at least eight steps, as schematically represented in **Figure 7**.

During these steps, the catalytic cycle may be interrupted and reactive oxygen species (ROS) are released, which can cause both DNA and other cellular damage that may lead to diseases [16] (adapted from [75]).

In general, the reaction course of mono-oxygenation catalysed by CYPs can be formulated by summary equation:

$$\mathbf{RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+}$$

Where 'RH' is a substrate and 'ROH' is its hydroxylated product of the reaction [4]

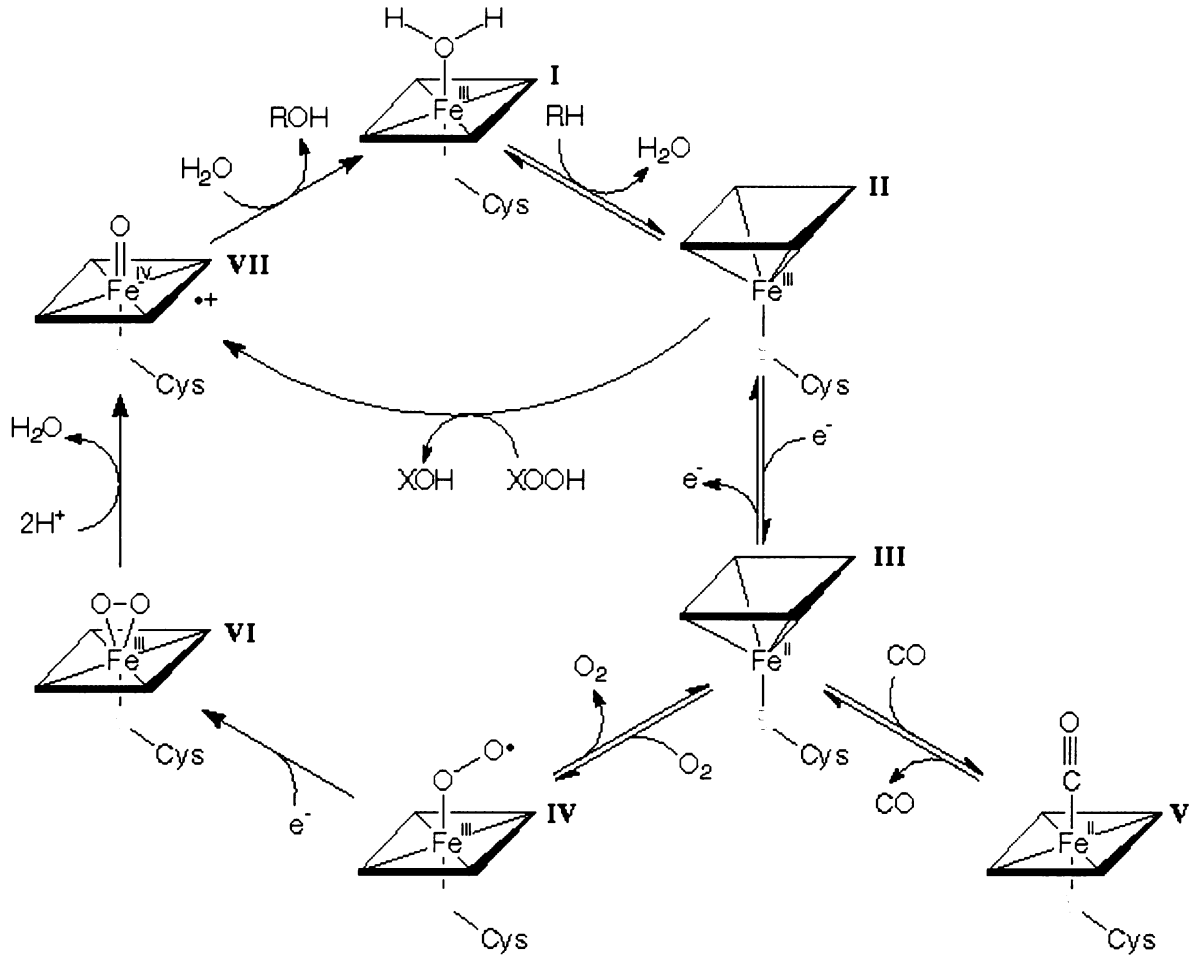


Figure 7 shows the reaction cycle scheme of mono-oxygenation reaction with the CYP catalytic cycle [28].

The cycle is initiated by substrate (RH) binding to the native, predominantly low-spin, hexacoordinate, ferric form **I**, converting it to the high-spin, pentacoordinate ferric complex **II**. One-electron reduction of **II** yields the high-spin, pentacoordinate ferrous complex **III**. Complex **III** subsequently binds dioxygen (O_2) to form a `semistable' low-spin, hexacoordinate ferrous-O_2 adduct **IV**. Species **I-IV** of the P450 cycle and the low-spin, hexacoordinate ferrous-CO inhibitor complex **V** have been isolated and well characterised. It is hypothesised that such species as a low-spin, ferric peroxycomplex **VI**, and oxyferryl (Fe^{IV}=O) intermediate **VII**, are further formed. Oxygen atom transfer from **VII** to the substrate yields oxidised product (ROH) and regenerates state **I**. In the presence of external oxygenation agents, such as organic peroxides (per-acids), the complex **III** may directly yield state **VII** *via* a `shunt' pathway [28].

2.4 Enzymes metabolizing the xenobiotics by reduction mechanism

The reduction reactions participate in the xenobiotics biotransformation in a lesser extent than oxidation reactions [21]. However, reduction represents an important pathway of nitro-aromatics biotransformation. Major xenobiotic's reductases, beside already mentioned NAD(P)H:cytochrome P450 reductase, are DT-diaphorase and xanthine oxidase

2.4.1 DT-DIAPHORASE

DT-diaphorase [NAD(P)H:quinone oxidoreductase, EC 1.6.5.2, NQO1] an obligate two-electron reducing enzyme, catalyzes the reduction of various quinones (particularly short-chain acceptors, e.g. ubiquinone, benzoquinone, juglone [29]) into hydroquinones [30] and can utilize either NADH or NADPH as an electron donor [31]:

$$\textbf{NAD(P)H + quinone} \rightarrow \textbf{NADP}^{+} \textbf{+ hydroquinone}$$ [29]

DT-diaphorase is a dimer of identical subunits, each comprising 273 amino acids [32]. It has one FAD prosthetic group, as a cofactor, in each subunit [14]. FAD is noncovalently attached to the subunit, but remains bound during catalytic cycling. Each subunit contains two separate domains [32]:

- a major catalytic domain (residues 1-220) folded in a predominantly α/β structure
- a small, C-terminal domain

As the structure of DT-diaphorase was determined, the two-electron reductions promoted by this enzyme were clearly explained: both halves of the reaction involve hydride transfer – first from NAD(P)H to FAD and then from $FADH_2$ to the quinone [32].

The toxic effects of quinones are based in their covalent binding to DNA, RNA, and proteins, and in their participation in one-electron oxidatively-reducing transfers leading to oxidative stress appearance [14].

Beside quinone reduction, DT-diaphorase can also protect cells against the toxic and neoplastic effects of free radicals and reacting oxygen species arising from one-electron reductions [32], including nitroaromatic reductions [14]. Moreover, DT-diaphorase also reductively

activates important chemotherapeutics, cytostatics, such as mitomycins and aziridylbenzoquinones. Thus, since levels of DT-diaphorase are elevated in tumours, the selective susceptibility of such agents provides the opportunity for designing improved chemotherapeutic agents that are more efficiently activated by this enzyme [32].

The majority of DT-diaphorase resides in the cytosolic fraction of the cell, with minor activity in mitochondria and endoplasmic reticulum [33].

2.4.2 XANTHINE OXIDASE

Xanthine oxidase (XO) belongs to the xanthine oxidase enzymes family, which contain a molybdenum cofactor (Moco) and [2Fe-2S] clusters and catalyse the oxidative hydroxylation of a range of aromatic heterocyclic compounds and aldehydes (RCHO) in reactions that involve the cleavage of a C-H bond [34].

The flavoprotein enzyme XO is a mini-electron-transport-protein [7], which catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid as shown in the equations below [35]:

$$\textbf{hypoxanthine} + O_2 + H_2O \rightarrow \textbf{xanthine} + H_2O_2$$

$$\textbf{xanthine} + O_2 + H_2O \rightarrow \textbf{uric acid} + H_2O_2$$

Eukaryotic xanthine oxidase is a homo-dimer of ~1330-residue subunits, each of which binds a variety of electron-transfer agents in its domains [7]:

- an FAD (flavin adenine dinucleotide)
- two spectroscopially distinct [2Fe-2S] clusters (ferredoxin iron-sulphur clusters)
- molybdenum cofactor (molybdopterin complex) in which the Mo cycles between its Mo(IV) and Mo(VI) oxidation states.

The X-ray structure of XO reveals that the FAD and the molybdopterin complex are interposed by the two [2Fe-2S] clusters to form a mini-electron-transport chain [7].

In humans, xanthine oxidase is normally found in the liver tissue in the form of xanthine-dehydrogenase, which can be transformed into xanthine oxidase by storing in -20°C temperature in organic solvents [14].

3. NITROAROMATICS

Nitroaromatics are important pollutants entering the environment primarily through anthropogenic activities associated with the industrial production of dyes, explosives, pesticides and pharmaceuticals [25]. Exposure to nitro-aromatic compounds occurs in a variety of ways. Polycyclic aromatic hydrocarbons react with nitrogen oxides to form nitro-aromatics under the conditions that might be expected in polluted air and in combustion processes. As a result, nitro-aromatic compounds are present in many mixtures such as cigarette smoke, fly and diesel exhaust [12]. Discharge of these compounds into the environment poses serious health hazards, as they are mutagenic and bioaccumulate in the food chain [25]. Nitro-aromatics are also carcinogens inducing oncogenic processes.

Whilst singly nitrated aromatic compounds are usually mineralised in the environment, multiply nitrated aromatics are recalcitrant and highly toxic [36] as multiple nitro groups in aromatics profoundly influence their binding to DNA, and thus their carcinogenic potential [12]. Moreover, aromatic compounds with multiple nitro substituents are known to be resistant to electrophilic attack by oxygenases. Most of the nitro-aromatics are also potent uncouplers of oxidative and photosynthetic phosphorylation [25].

3.1 Parent compounds of nitroaromatics

3.1.1 BENZANTHRONE

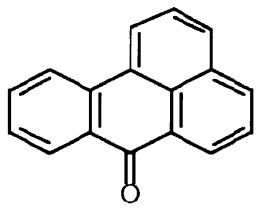


Figure 8: Constitution formula of benzanthrone

Benzanthrone (7H-benz[d,e]anthracen-7-one) (**Figure 8**) is used for synthesis of many dyes (e.g. Yellow 4, Green 1) and it also serves as an intermediate to combine with other dyes.

Epidemiological studies indicate that skin, respiratory, gastrointestinal, genitourinary, and nervous and haemopoietic systems are affected by benzanthrone exposure. [16] (adapted from [76]).

Benzanthrone can also cause photosensitivity and many of its substituted analogues are also health hazardous [16] (adapted from [77, 78]). Experimental results indicate that toxicity caused by benzanthrone-derived dyes or dye intermediates appear to be influenced by the number of carbonyl and aminoanthraquinone groups as well as by the presence of functional groups (like halogen, nitro, hydroxy and methoxy) attached to the parent molecule, benzanthrone [16] (adapted from [77]).

Although most of these benzanthrone dyes are produced through synthesis, there are experiments suggesting that modification of parent compound could occur under certain atmospheric conditions [16] (adapted from [79]). For instance, nitro-derivatives can be formed by the hydroxyl radical-initiated reactions in the presence of ozone (O_3), nitrate radical-initiated reactions and photolysis. Furthermore, rearrangements in the atmosphere can also give rise to new isomers [16] (adapted from [80]).

3.1.2 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are generally divided into two categories based on their structures; these are called *peri-* or *cata*-condensed.

- Peri-condensed structures have the characteristic feature that they can form cycles and can further be subdivided into two classes [16] (adapted from [81]).
- Cata-condensed systems are always formed by six-membered rings (they are *alternants*) and do not form cycles [16] (adapted from [81]).

The structure of single PAHs, such as carbon atom positions or structure regions, can also be divided into several categories, which in turn determine the reactivity of the substance itself [16] (adapted from [82]). This is illustrated by benzo[a]pyrene (BaP) and dibenzo[a,l]pyrene in **Figure 9**.

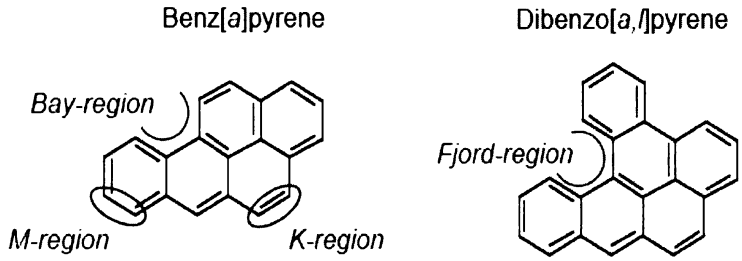


Figure 9: Structure regions of PAHs represented by benzo[a]pyrene and dibenzo[a,l]pyrene [16] (adapted from [81]).

The **'K'-region** is defined as the external corner of the molecule, whereas the **'Bay'-region** is an open inner corner. The **'M'-region** is also referred to as distal Bay-region. The **'Fjord'-region** is a sterically hindered aromatic region and is of great interest because of its significantly greater tumorigenic activity compared to the planar Bay-region derivatives. BaP can form a Bay-region diol-epoxide, and analogous diol-epoxides are now recognised as tumorigenic metabolites of numerous PAHs [16] (adapted from [81]).

PAHs belong to the group of contaminants that can accumulate to toxic levels in the body within a short period and have been implicated of being causative agents of cancer in humans [16]. It is not only the structure of PAH molecule that greatly influences the harmfulness of the compound, but also the position and orientation of such functional groups as amino-, nitro-, hydroxy-, halogen-, or alkyl-groups. For instance, studies have shown that dinitropyrenes (DNP) are more genotoxic than nitropyrenes (NP), and that there is a structure-activity hierarchy too [16] (adapted from [83]).

3.2 Metabolism of nitroaromatics

As mentioned in previous chapter ("Biotransformation of carcinogens"), most of the carcinogens require metabolic activation to form DNA adducts. The crucial location of nitro-aromatics metabolic activation is nitro group [37]. These compounds are readily reduced by anaerobic consortia to their respective amines [25]. Nitro reduction gives rise to hydroxylamine, which creates a nitrenium ion forming DNA adducts [37]. Oxidative metabolism is conductive to

the nitroaromatics bioactivation too but not in such a degree. It is suspected that nitroaromatics can further modify DNA non-covalently, via radical process initiation. (**Figure 10**)

Nitrated polycyclic hydrocarbons, and those nitroaromatics mentioned above will be described more in detail in the following text.

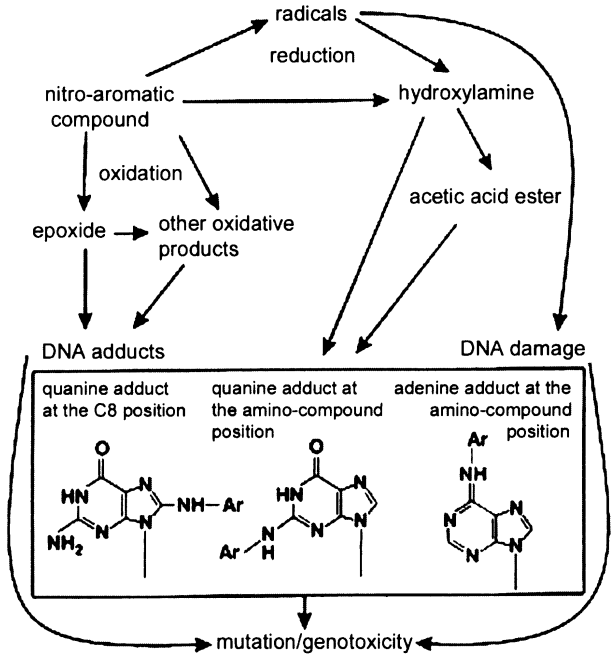


Figure 10: Summary of metabolism of nitroaromatics and the DNA damage caused by them [37]

3.3 Nitroaromatics and human health

National Toxicology Program in the USA, listed the following substances as strong potent carcinogens to the humans organism: 2-nitroanisole, 1-nitropyrene, 4-nitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene, 6-nitrochrysene and nitrophene. 2-nitrofluorene has been yet added to that list of strong potent carcinogens by International Agency for Research on Cancer (IARC). Another physiologically effective nitroaromatics like aristolochic acid herbal remedies, nitrofuranes and nitroimidazoles widely used in both human and veterinary medicine, should be noted, too [12].

In many studies there has been provided the genotoxicity of nitroaromatics that have been identified in diesel exhaust and ambient air particulates e.g. 3-nitrobenzanthrone and its metabolites, 2-nitropyrene, 2-nitrofluoranthene and 1,6- and 1,8- dinitropyrene [38].

3.3.1 NITRATED POLYCYCLIC AROMATIC HYDROCARBONS

Nitrated polycyclic aromatic hydrocarbons (Nitro-PAH) are widely distributed environmental pollutants found in vehicular exhaust from diesel and gasoline engines and on the surface of ambient air particulate matter [2]. Many members of this class of compounds are known to be mutagenic in bacterial and mammalian cells and tumorigenic in rodents and some are probably carcinogenic to humans [39].

As mentioned above, nitro-PAHs require metabolic activation to form electrophilic species to exert their genotoxic activity. The activation of nitro-PAHs is mainly through nitro-reduction (**Figure 11**) catalysed primarily by several cytosolic reductases, such as xanthine oxidase, DT-diaphorase and aldehyde oxidase [40], whereas CYP enzymes are primarily responsible for the oxidative metabolism of these compounds.

$$\text{R-NO}_2 \xrightarrow{[2H]} \text{R-NO} \xrightarrow{[2H]} \text{R-NHOH} \xrightarrow{[2H]} \text{R- NH}_2$$

Figure 11: Nitro-reduction in the body is catalysed primarily by cytosolic reductases [40].

However, the reductive activation of nitro-aromatics including 3-NBA by CYP enzymes has also been demonstrated [3]. *N*-hydroxy arylamine intermediates, formed by reduction of nitro-PAHs, can further be metabolized by phase II enzymes, such as *N,O*-acetyltransferases (NATs) or sulfotransferases (SULTs), leading to the formation of reactive esters (e.g. *N*-acetoxy or *N*-sulfoxy arylamines), which undergo heterolysis of the N-O or S-O bond to produce electrophilic nitrenium ion capable of reacting with DNA to form DNA adducts [41]. Therefore, NAT and SULT expression in the human respiratory system could contribute significantly and specifically to the metabolic activation of 3-nitrobenzanthron. Bioactivation of aromatic hydroxylamines has

been primarily observed with human SULT1A1 and SULT1A2, but various *N*-hydroxy arylamines in humans are also catalysed by two isomers of NAT, designed NAT1 and NAT2 [39].

3.3.2 3-NITROBENZANTHRONE

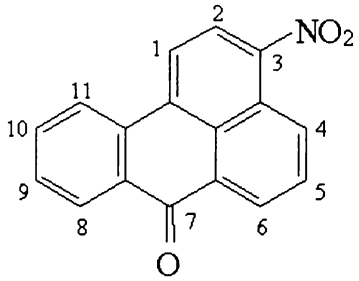


Figure 12: The structure of 3-nitrobenzanthrone

3-nitrobenzanthrone (3-NBA, 3-nitro-7H-benz[d,e]anthracen-7-one) is a nitro-derivative of ketone benzanthrone that has profound effects on biological systems [42]. It was first isolated from diesel exhaust and airborne particles in 1997 [43]. The structure of 3-NBA is shown in **Figure 12.**

Among the 3-NBA isomers, 9- and 11-NBA are also mutagenic and 2-NBA is easily formed from the gas-phase reaction of BA and NO_3 or OH radical in the presence of NO_2 in the atmosphere. The NBA isomers are weakly basic because the oxygen atom of benzanthrone (at position 7) has two lone pairs [44].

3-NBA is a rodent carcinogen, and suspected human carcinogen and also a potent mutagen identified in diesel exhaust and in airborne particular matter. 3-NBA might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere (benzanthrone with NO_2 and O_3). Furthermore, 3-NBA has also been detected recently in surface soil and rainwater, as a likely consequence of atmospheric washout [45]. 3-NBA was characterized as an exceptional potent direct-acting mutagen in mutagenicity tests (Ames *Salmonella typhimurium* assay and transgenic Muta Mouse assay) [16], with a mutation frequency comparable to that of 1,8-dinitropyrene, a direct acting mutagen which has the highest activity thus far [38].

3-NBA has also been shown to be genotoxic in several short-term tests. Its genotoxicity has been further documented by the detection of specific DNA adducts formed *in vitro* as well as *in vivo* in rodents [45].

3-NBA induces several different types of mutations, such as frameshift, transversions, and transitions, and produces a high level of DNA adducts in vivo, as well as tumors and oxidative damage [42].

Metabolic activation of 3-NBA

Nitroreduction catalyzed by cytosolic and microsomal nitroreductases (NQO1, CYPs) followed by *O*-acetylation catalysed by *N*-acetyltransferases (NATs) and/or *O*-sulfonation catalyzed by sulfotransferases (SULTs), seem to be the major pathways of bioactivation for 3-NBA leading to DNA adduct formation [46]. **Figure 13** demonstrates the major pathway of 3-NBA metabolism. - NO_2 group is reduced into N-hydroxylamine (-NHOH), which is unstable and forms a nitrenium ion, as mentioned in previous section. Arylnitrenium ions and rearranged carbenium ions react with nucleophile DNA centers, leading to the formation of purine adducts at the C8 and N^2 position of guanine and at the C8 and N^6 position of adenine [43].

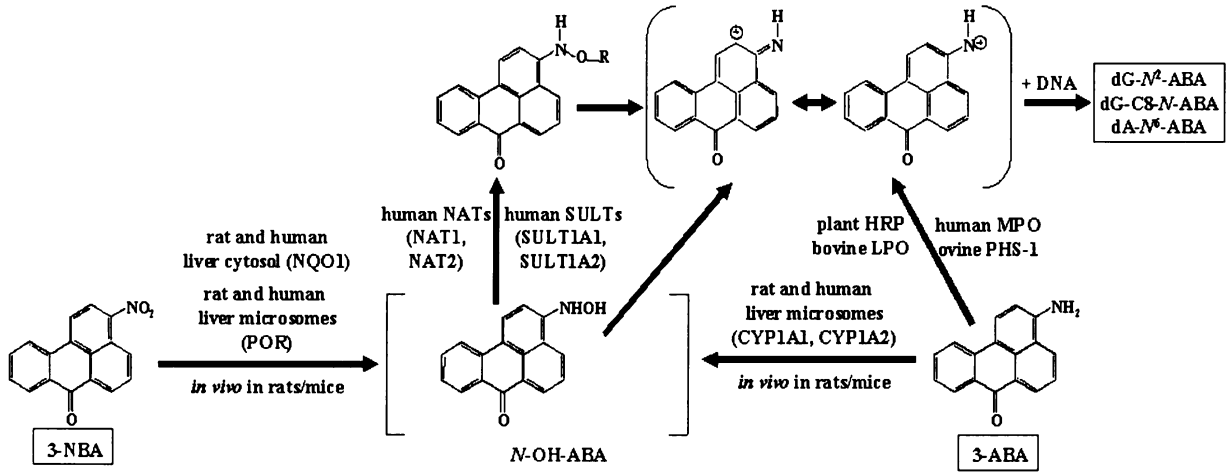


Figure 13: Pathways of metabolic activation and DNA adduct formation of 3-nitronenzanthrone and 3-aminobenzanthrone. HRP, horseradish peroxidase, PHS-1, prostaglandin H-synthase 1. Other abbreviations are shown in the text and in the List of abbreviations.

Most of the metabolic activation of 3-NBA *in vitro* (reductive bio-activation) is attributable to human and rat cytosolic NQO1, whereas human NAT2, followed by NAT1, SULT1A1 and to a lesser extent, SULT1A2 are the major phase II enzymes activating 3-NBA [2].

Microsomal NADPH:cytochrome P450 reductase, an enzyme known to activate nitroaromatic compound, is also effective in the activation of 3-NBA, but in a model organism, mice, 3-NBA is predominantly activated by cytosolic nitroreductases such as NQO1 [2].

The DNA strand-breaking activity by 3-NBA and its metabolites has also been observed as measured by the Comet assay, so it also suggests that 3-NBA-DNA adducts are recognized by the nucleotide excision repair system [38].

Previously taken studies suggested that N-hydroxy-3-aminobenzanthrone (N-OH-ABA) appeared to be the critical intermediate for the formation of electrophilic arylnitrenium ions capable of reacting with DNA as mentioned above [46]. N-OH-ABA has also an ability to cause oxidative DNA damage that is dramatically enhanced by the endogenous reductant NADH [43].

The main metabolite of 3-NBA, **3-aminobenzanthrone** (3-ABA), has been detected in urine samples of smoking and non-smoking salt mine workers occupationally exposed to diesel emissions. So it is demonstrated that human exposure to 3-NBA in diesel emissions can be significant and is detectable [49]. In addition, 3-ABA is used industrially for colouring of microporous polyethylene films, which are widely used to separate liquid mixtures, in particular, in chemical batteries [16] (adapted from [84]). Moreover, the bright colour of 3-ABA is also suitable as a dye for textiles, daylight fluorescent pigments and laser dyes [16] (adapted from [85]). Furthermore, even though the epidemiological study on the toxicity of 3-ABA has not yet been evaluated, formation of DNA adducts by this reductive metabolite of 3-NBA *in vitro* and *in vivo* in rodents indicates its potential genotoxicity [46].

The results of Arlt et al [46] showed that both CYPs and peroxidases may play an important role in the oxidative metabolism of 3-ABA to reactive species that form DNA adducts, resulting in genotoxicity of 3-ABA and its parent compound [2] (**Figure 13**). It has been shown the correlation of CYP-linked enzyme activities with the level of DNA adducts found in rodents treated with 3-ABA, which has indicated that the most of the hepatic activation of 3-ABA was attributed to CYP1A1 and 1A2. Moreover, it was found that 3-ABA forms the same DNA adducts as 3-NBA *in vitro* and *in vivo* in rodents [2, 46].

Besides CYP enzymes, in tissues with low expression of CYP enzymes, 3-ABA might be activated by other enzymes. Peroxidases (**Figure 13**) are known to be involved in the metabolic activation of various procarcinogens including aromatic and heterocyclic amines [46].

Peroxidases such as prostaglandin H synthase [cyclooxygenase (COX)], lactoperoxidase (LPO) and myeloperoxidase (MPO), abundant in several extrahepatic tissues, generate DNA-adducts, which are formed *in vivo* by 3-ABA or 3-NBA [2, 46].

3.3.3 2-NITROBENZANTHRONE

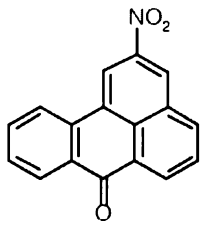


Figure 14: 2-Nitrobenzanthrone

2-Nitrobenzanthrone (2-NBA) is an isomer of nitrobenzanthrone, and results from extensive rearrangement of 3-NBA in the atmosphere.

In vitro studies have shown that in human cell models (lung and hepatocyte cell lines), 2-NBA is about 30-50% as genotoxic compared to 3-NBA in terms of oxidative stress and DNA adduct formation, but exists at 70-fold higher concentrations than 3-NBA in ambient air [16] (adapted from [42]).

The amount of DNA lesions produced by 2-NBA is not as high as those formed due to 3-NBA exposure, but 2-NBA is more lipophilic, hence it may be metabolized more slowly and thereby subjecting the body to reactive metabolites for a longer period of time compared to 3-NBA [16] (adapted from [86]).

3.3.4 2-NITROANISOLE

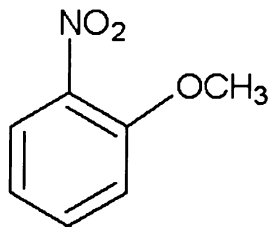


Figure 15: 2-Nitroanisole

2-Nitroanisole (1-methoxy-2-nitrobenzene, 2-NA) (**Figure 15**) is an important industrial pollutant and a potent bladder carcinogen for rodents [49].

2-NA is used primarily as a precursor in the synthesis of 2-methoxyaniline (*o*-anisidine), an intermediate in the manufacture

of many azo dyes and naphtol pigments, which are used for printing and for paper and textile dyeing [51]. 2-NA and *o*-anisidine exhibit strong carcinogenic activity, causing neoplastic transformation in the urinary bladder, and to a lesser extent, in spleen, liver and kidneys in rodents [52]. 2-NA is also a toxic compound, causing anaemia, characterised by increased levels of methemoglobin and accelerated destruction of erythrocytes [53].

It has not been determined exactly whether 2-NA is a genotoxic or epigenetic carcinogen [50] and in which phase of carcinogenesis it is involved [53]. In spite of potent rodent carcinogenicity of 2-NA, this chemical is weakly mutagenic in the Ames thest with the *Salmonella typhimurium* TA100 strains. This carcinogen also exhibits a low activity in cytogenetic tests. It induces a slight increase in chromosomal aberrations and in sister chromatid exchanges, but only at high concentrations. An explanation for these discrepancies could be different enzyme patterns, which are responsible for 2-NA activation or detoxication in different cells, organs and species [41].

Metabolic activation of 2-NA

Xanthine oxidase is the principal enzyme responsible for the reductive metabolism of 2-NA in cytosol, catalyzing the formation of N-(2-methoxyphenyl)hydroxylamine and *o*-anisidine [52], which are two major products generated in these reactions [41] leading to form DNA adducts. The final reductive metabolite of 2-NA is *o*-anisidine [51].

Deoxyguanosine adducts derived from N-(2-methoxyphenyl)hydroxylamine were found *in vivo* in DNA of several tissues of rats treated with 2-NA as well as *in vitro* [52].

In contrast, 2-NA oxidation by microsomal CYP enzymes from human, rat and rabbit to 2-nitrophenol (**Figure 16**), which appears in urine predominantly as the sulphate conjugate or as the glucuronic acid conjugate [49], and hydroxyderivatives of this metabolite leads to its detoxification [52].

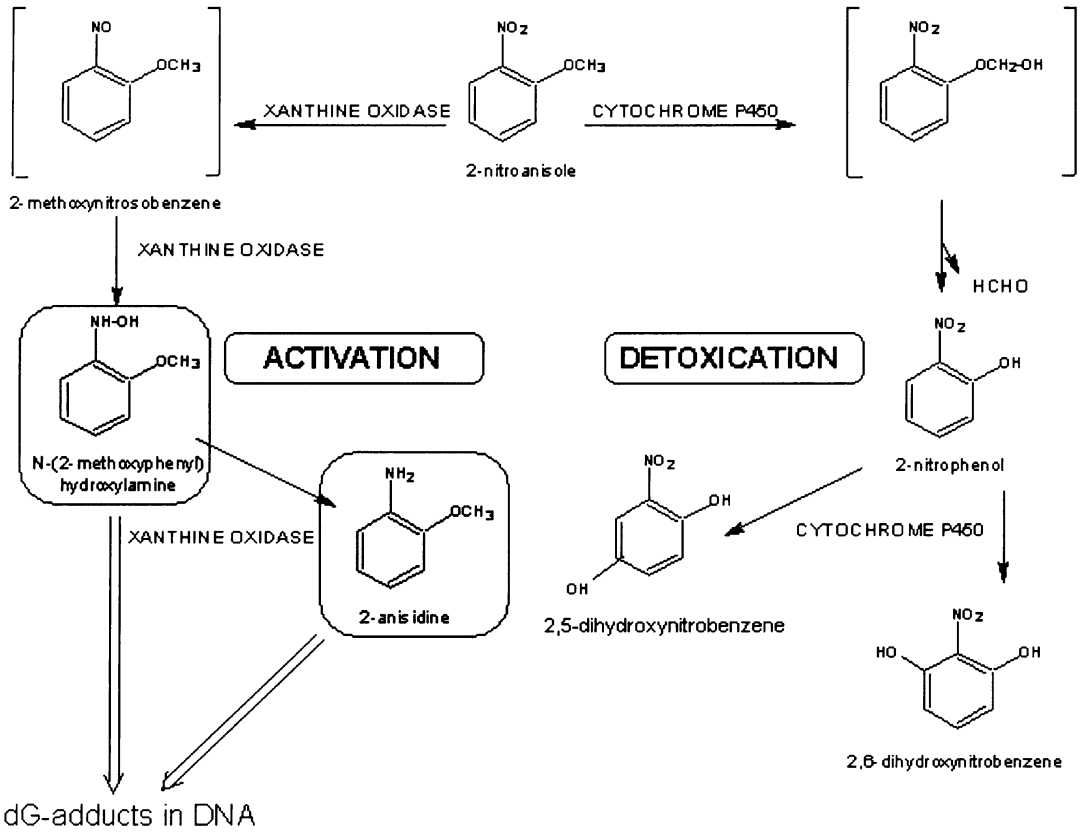


Figure 16: Metabolic activation of 2-nitroanisole.

Recently, it was found that ***o*-anisidine**, reductive metabolite of 2-nitroanisole, is oxidatively activated by peroxidases *in vitro* to species binding to DNA, which suggests a genotoxic mechanism of *o*-anisidine carcinogenicity. Nevertheless, knowledge on the *in vivo* DNA adduct formation by *o*-anisidine is necessary to confirm a genotoxic metabolism [51].

o-Anisidine is also oxidized like other aromatic amines to a reactive *N*-hydroxyarylamine intermediate [54].

N-hydroxyarylamine intermediates of carcinogenic aromatic amines can further be metabolised by phase II enzymes (NATs, SULTs) leading to the formation of reactive esters, *e.g.*, *N*-acetoxy- or *N*-sulfooxyarylamine, which undergo hydrolysis of the N-O or S-O bond to produce electrophilic nitrenium ions capable of reacting with DNA to form DNA adducts [52].

3.3.5 ARISTOLOCHIC ACID

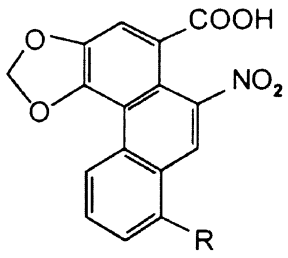


Figure 17: Aristolochic acid I: R = OCH_3
Aristolochic acid II: R = H

Aristolochic acid (AA), the plant extract of *Aristolochia* spp., is a mixture of structurally related nitrophenanthrene carboxylic acids, mainly aristolochic acid I (AAI) and aristolochic acid II (AAII) [55] which differ only by one methoxy group, being the major component.(**Figure 17**) [56].

Herbal drugs derived from *Aristolochia* spp. have been known since antiquity. Contemporary medicine has used *Aristolochia* plant extracts for the therapy of arthritis, gout, rheumatism and festering wounds. The development of pharmaceutical preparations showed that AA is a strong carcinogen in rats. Subsequently, AA was shown to be a genotoxic mutagen in several short-term tests. Although all pharmaceutical preparations containing AA have been withdrawn from the market in many countries, *Aristolochia* plants and their extracts have been further used in traditional medicine in some parts of the world [55].

So-called Chinese herbs nepropathy (CHN), a unique type of rapidly progressive renal fibrosis associated with the prolonged intake of Chinese herbs during a slimming regimen, was observed for the first time in Belgium in 1991 [57]. The old herbal drug aristolochic acid has also been associated with the development of novel nephropathy, designated the interstitial aristolochic acid nephropathy (AAN) [57], and urothelial cancer in AAN patients found recently in the south Europe countries. This highlights the carcinogenic potential of AA begins. It has been suggested that DNA damage by AA is not only responsible for the tumour development but also for the destructive fibrotic process in the kidney.

AA is a powerful nephrotoxic and carcinogenic substance with an extremely short latency period too, not only in animals but also in humans, forming covalent DNA adducts [55].

Metabolic activation of AA

Both AAI and AAII are genotoxic mutagens forming DNA adducts after metabolic activation through simple reduction of the nitro group, under unaerobic conditions. The activating

metabolism has been elucidated and is consistent with the formation of a cyclic nitrenium ion with delocalized charge leading to the preferential formation of purine adducts bound to the exocyclic amino groups of deoxyadenosine and deoxyguanosine as shown in **Figure 18** [55].

Both microsomal and cytosolic enzymes (e.g. CYPs, DT-diaphorase, XO, peroxidases such as ovine prostaglandin H synthase, human NQO1) activate AAI and AAII to form the same DNA adducts found *in vivo* in rodents and in humans suffering from AAN [57]. The predominant DNA adduct *in vivo,* 7-(deoxyadenosin-N^6-yl)aristolactam I is the most persistent of the adducts in target tissue, and is a mutagenic lesion too [55].

The final products of nitroreduction, the corresponding **aristolactams**, are the major metabolites found in urine and faeces [57]. Aristolactams are not mutagenic themselves and require metabolic activation by an exogenous metabolic system [57].

The studies have shown, that under aerobic conditions, AAI is extensively demethylated into aristolic acid I, to its major product, while AAII remains unaltered.

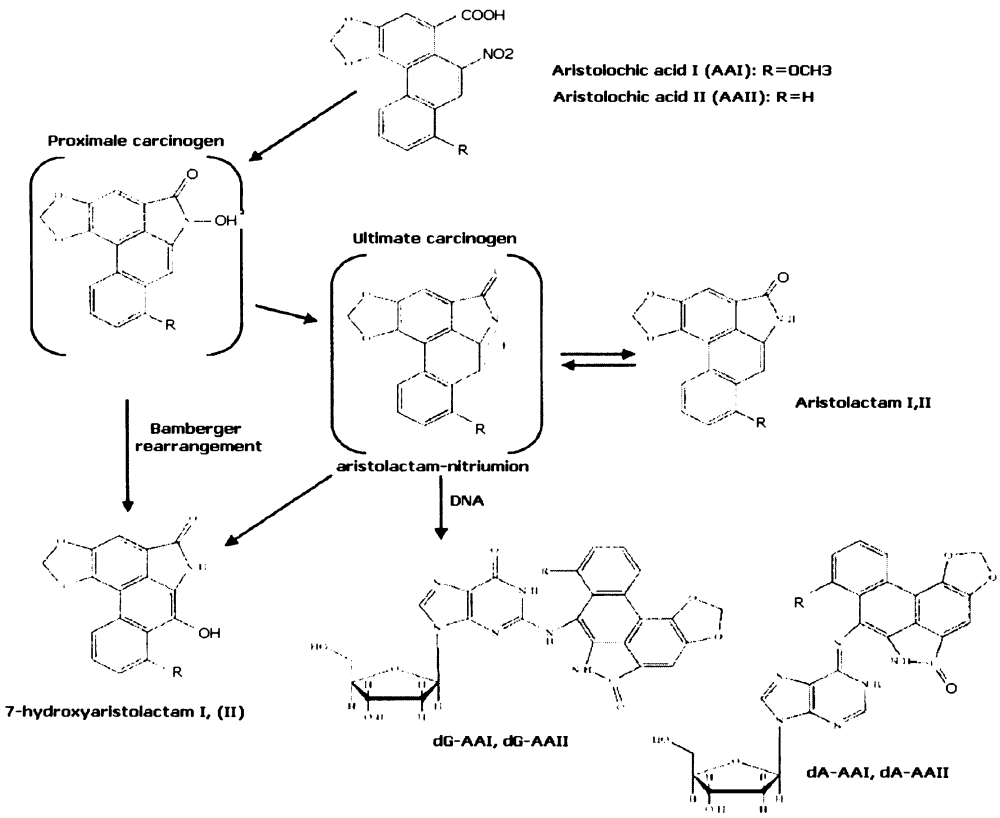


Figure 18: Metabolic activation and DNA adduct formation of aristolochic acid I (R = OCH_3) and II (R = H) [57].

3.3.6 NITROPYRENES

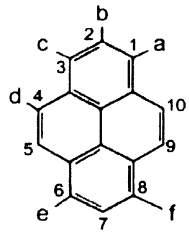


Figure 19: Nitropyrenes; a-f are possible positions for -NO2

Nitrated pyrenes (**Figure 19**) comprise a group of environmental contaminants [58]. Most of the nitropyrenes are mutagenic and tumorigenic, but their potency sometimes differs by more than an order of magnitude [59].

Mono-Nitropyrenes (Mono-NPs) shown in **Figure 20** have been found in various environmental matrices [60] such as in diesel exhaust particulate matter, coal fly ash, exhaust water from gasoline stations [61]. Among the three mono-NP isomers, 1-Nitropyre (1-NP) is the most prevalent. In urban air, the levels of 2- and 4-Nitropyrene (2-NP and 4-NP) are comparable but much lower than that of 1-NP [60].

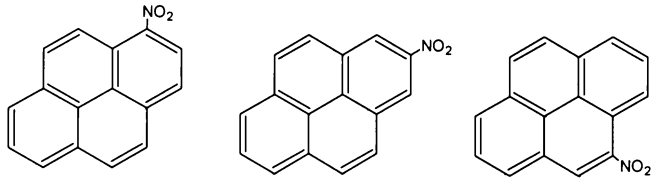


Figure 20: Structures of 1-NP, 2-NP and 4-NP

1-NP is the predominant nitro-PAH found in diesel exhaust [12], is both mutagen and a tumorigen, and it may account for up to 25% of the mutagenic activity in diesel particulate [58]. The presence of 1-NP in lung specimens of lung cancer patients has been detected as a result of inhaling soot from the combustion of coal and heavy oil used for cooking (1-NP was detected in grilled chicken) and indoor heating [60]. 2-NP is detected in the atmosphere and it is believed to be formed by photochemical processes involving the reaction of pyrene with peroxy-nitrogen derivatives [62]. 4-NP apparently exists only as a synthetic product [62].

As mentioned before, the position of the nitro group determines the mutagenic and carcinogenic activities of mono-NPs: [60]

- In bacterial systems, 2-NP is more mutagenic than 1-NP, whereas the mutagenic activity of 4-NP exceeds that of 2-NP [60].
- In *S. Typhimurium* mutagenicity test 2- and 4- isomers were being 10 to 20 times more mutagenic than the 1- isomer in the same strain [62].

Metabolic activation of nitropyrenes

Nitroreduction is a major pathway of bioactivation of all nitropyrenes, whereas *O*-esterification enzymes, in addition, play a crucial role in the mutagenicity of dinitropyrenes [59]. Nitro-group is reduced into unstable hydroxylamine, which readily forms a nitrenium ion leading to the formation of DNA adducts.

To the nitropyrenes activation also contributes their oxidative metabolism yielding to form epoxides as shown in **Figure 21** [12]. However, those oxidative products appear in much lesser extent. Oxidation of these compounds leads rather to their detoxification [12].

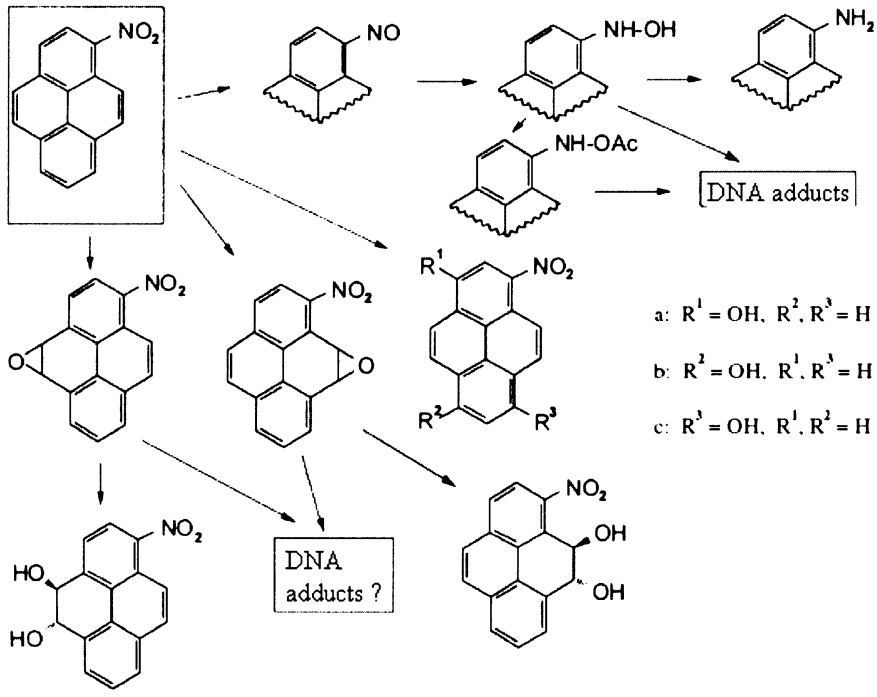


Figure 21: Metabolic activation of 1-nitropyrene

The metabolism of 1-NP has been shown to involve both CYP-mediated C-oxidation and nitroreduction. The C-oxidation of 1-NP by CYP can result into the formation of two arene K-region oxides, and two 1-nitropyrene oxides. The K-region oxides can be further hydrolised by epoxide hydrolase to the corresponding K-region dihydrodiols or rearranged to form four K-region phenols. Alternatively, CYP can catalize the direct formation of three phenols with different reported mutagenicity [63].

In *Salmonella typhimurium*, 1-NP is mutagenic through nitroreduction to the corresponding nitroso then the hydroxylamino derivative, a mutagenic metabolite of 1-NP, which has been shown to form a C8 guanyl adduct [53].

Dinitropyrenes (DNPs) substituted in the positions 1,3-, 1,6- and 1,8- (**Figure 21**) [64] are the minor components in diesel exhaust, but they account for 30% or more of the mutagenic activity of diesel particulate [58]. DNPs, especially 1,8-dinitropyrene (1,8-DNP) and 1,6-dinitropyrene (1,6-DNP), are much more potent mutagens and carcinogens than other nitropyrenes [64].

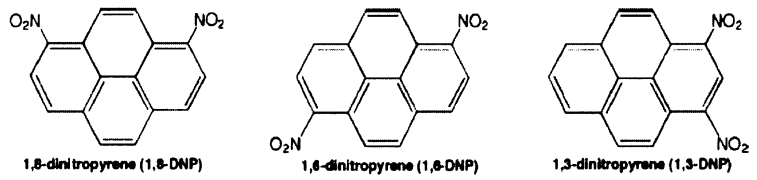


Figure 21: Chemical structures of DNPs

Several dinitropyrenes, such as 1,3-, 1,6- and 1,8-dinitropyrenes, show strong direct-acting mutagenicity by the Ames test using *Salmonella typhimurium* strains [44]. Moreover, DNPs also induced lung cancer and leukemia in rodents, and IARC has assessed that 1,8-DNP and 1,6-DNP have been possibly carcinogenic to humans [64].

Metabolic activation of dinitropyrenes

DNA adduct formation after metabolic activation has been concidered to be a major casual factor of carcinogenesis by DNPs [64]. DNPs undergo nitroreduction to N-hydroxy-arylamines that bind to DNA directly or after *O*-esterification. DNP adducts are identified as N-(deoxyguanosin-8-yl)amino-nitropyrene, leading to mutation and carcinogenesis [64].

CONCLUSION

The aim of this work was to summarise facts and information about various nitroaromatic compounds from the research sources, in order to extend and unify our knowledge regarding occurrence of nitroaromatics and their metabolism.

The attention was mainly focused on 3-nitrobenzanthrone and 2-nitroanisole, strong carcinogens and mutagens for rats and potential carcinogens for humans.

The research of nitro-aromatic compounds is important mainly because of their occurrence in the environment, as pollutants, and because of their toxicity. Nitroaromatic compounds are also utilised in prodrug strategies in cancer therapy as tumour activated prodrugs.

It is obvious that the environment significantly influence human diseases. Diseases caused by polluted environment can be diagnosed in human organism immediately in the early stage of the disease, thereby prevent the disease from spreading.

Elucidation of the biotransformation of nitroaromatics, including the enzymes involved in their bioactivation, and the final product of their biotransformation is crucial for understanding of their impact in human organism. Nitroreduction catalysed by cytosolic and microsomal nitroreductases has been ascertained to be the major pathway of biotransformation of the nitroaromatic compound mostly leading to DNA adducts formation.

Further development in this area in future can be expected.

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