

## 4.2: Toxicodynamics and Molecular Interactions

### 4.2. Toxicodynamics & Molecular Interactions

**Author:** Timo Hamers

**Reviewers:** Frank van Belleghem and Ludek Blaha

#### Learning goals

You should be able to

- explain that a toxic response requires a molecular interaction between a toxic compound and its target
- name at least three different types of biomolecular targets
- name at least three functions of proteins that can be hampered by toxic compounds
- explain in general terms the consequences of molecular interaction with a receptor protein, an enzyme, a transporter protein, a DNA molecule, and a membrane lipid bilayer.

**Key words:** Receptor; Transcription factor; DNA adducts; Membrane; Oxidative stress

#### Description

Toxicodynamics describes the dynamic interactions between a compound and its biological target, leading ultimately to an (adverse) effect. In this Chapter 4.2, toxicodynamics have been described for processes leading to diverse adverse effects. Any adverse effects by a toxic substance is the result of an interaction between the toxicant and its biomolecular target (i.e. mechanism of action). Biomolecular targets include a protein, a DNA or RNA molecule, a phospholipid bilayer membrane, but also small molecules that have specific functions in keeping cellular homeostasis.

Both endogenous and xenobiotic compounds that bind to proteins are called ligands. The consequence of a protein interaction depends on the role of the target protein, e.g.

1. Receptor
2. Enzyme
3. Protein

Receptor proteins specifically bind and respond to endogenous signalling ligands such as hormones, prostaglandins, growth factors, or neurotransmitters, by causing a typical cellular response. Receptor proteins can be located in the cell membrane, in the cytosol, and in the nucleus of a cell. Agonistic receptor ligands activate the receptor protein whereas antagonistic ligands inactivate the receptor and prevent (endogenous) agonists from activating the receptor. Based on the role of the receptor protein, binding by ligands may interfere with ion channels, G-protein coupled receptors, enzyme linked receptors, or nuclear receptors. Xenobiotic ligands can interfere with these cellular responses by acting as agonistic or antagonistic ligands (link to section on [Receptor interaction](#)).

Compounds that bind to an enzyme usually cause inhibition of the enzyme activity, i.e. a decrease in the conversion rate of the endogenous substrate(s) of the enzyme into its/their corresponding product(s). Compounds that bind non-covalently to an enzyme cause reversible inhibition, while compounds that bind covalently to an enzyme cause irreversible inhibition (link to section on [Protein inactivation](#)).

Similarly, compounds that bind to a transporter protein usually inhibit the transport of the natural, endogenous ligand. Such transporter proteins may be responsible for local transport of endogenous ligands across the cell membrane, but also for peripheral transport of endogenous ligands through the blood from one organ to the other (link to section [Endocrine disruption](#)).

Apart from interaction with functional receptor, enzyme, or transporter proteins, toxic compounds may also interact with structural proteins. For instance the cytoskeleton may be damaged by toxic compounds that block the polymerization of actin, thereby preventing the formation of filaments.

In addition to proteins, DNA and RNA macromolecules can be targets for compound binding. Especially the guanine base can be covalently bound by electrophilic compounds, such as reactive metabolites. Such DNA adducts may cause copy errors during DNA replication leading to point mutations (link to section on [Genotoxicity](#)).

Compounds may also interfere with phospholipid bilayer membranes, especially with the outer cell membrane and with mitochondrial membranes. Compounds disturb the membrane integrity and functioning by partitioning into the lipid bilayer. Lost membrane integrity may ultimately lead to leakage of electrolytes and loss of membrane potential.

#### Narcosis and Membrane Damage

Partitioning into the lipid bilayer is a non-specific process. Therefore, concentrations in biological membranes that cause effects through this mode of action do not differ between compounds. As such, this type of toxicity is considered as a "baseline toxicity" (also called "narcosis"), which is exerted by all chemicals. For instance, the chemical concentration in a target membrane causing 50% mortality in a test population is around 50 mmol/kg lipid, irrespective of the species or compound under consideration. Based on external exposure levels, however, compounds do have different narcotic potencies. After all, to reach similar lipid-based internal concentrations, different exposure concentrations are required, depending on the lipid-water partitioning coefficient, which is an intrinsic property of a compound, and not of the species.

Narcotic action is not the only mechanism by which compounds may damage membrane integrity. Compounds called "ionophores", for instance, act like ion carriers that transport ions across the membrane, thereby disrupting the electrolyte gradient across the membrane. Ionophores should not be confused with compounds that open or close ion channels, although both type of compounds may disrupt the electrolyte gradient across the membrane. The difference is that ionophores dissolve in the bilayer membrane and shuttle transport ions across the membrane themselves, whereas ion channel inhibitors or stimulators close or open, respectively, a protein channel in the membrane that acts as a gate for ion transport.

Finally, it should be mentioned here that some compounds may cause oxidative stress by increasing the formation of reactive oxygen species (ROS), such as  $\text{H}_2\text{O}_2$ ,  $\text{O}_3$ ,  $\text{O}_2^{\cdot-}$ ,  $\cdot\text{OH}$ ,  $\text{NO}\cdot$ , or  $\text{RO}\cdot$ . ROS are oxygen metabolites that are found in any aerobic living organism. Compounds may directly cause an increase in ROS formation by undergoing redox cycling or interfering with the electron transport chain. Alternatively, compounds may cause an indirect increase in ROS formation by interference with ROS-scavenging antioxidants, ranging from small molecules (e.g. glutathione) to proteins (e.g. catalase or superoxide dismutase). For compounds causing both direct or indirect oxidative stress, it is not the compound itself that has a molecular interaction with the target, but the ROS which may bind covalently to DNA, proteins, and lipids (link to section on [Oxidative Stress](#)).

#### 4.2. Question 1

Name three biomolecular targets that can be affected by a compound

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#### 4.2. Question 2

Name three different mechanisms by which a compound can affect analyte transport across the cell membrane

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#### 4.2. Question 3

What is the difference between a receptor agonist and a receptor antagonist?

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#### 4.2.1. Protein Inactivation

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#### Learning objectives:

You should be able to

- discuss how a compound that binds to a protein may inhibit ligand binding, and thereby hamper the function of the protein
- explain the mechanism of action of organophosphate insecticides inhibiting acetylcholinesterase
- explain the mechanism of action of halogenated phenols inhibiting thyroid hormone transport by transthyretin
- distinguish between reversible and irreversible protein inactivation
- distinguish between competitive, non-competitive, and uncompetitive enzyme inhibition

**Key words:** enzyme inhibition; acetylcholinesterase, transthyretin, competitive inhibition, non-competitive inhibition, uncompetitive inhibition

## Introduction

Proteins play an important role in essential biochemical processes including catalysis of metabolic reactions, DNA replication and repair, transport of messengers (e.g. hormones), or receptor responses to such messengers. Many toxic compounds exert their toxic action by binding to a protein and thereby disturbing these vital protein functions.

## Inhibition of the protein transport function

Binding of xenobiotic compounds to a transporter protein may hamper binding of the natural ligand of the protein, thereby inhibiting the transporter function of the protein. An example of such inhibition is the binding of halogenated phenols to transthyretin (TTR). TTR is a transport protein for thyroid hormones, present in the blood. It has two binding places for the transport of thyroid hormone, i.e. mainly thyroxine (T4) in mammals and mainly triiodothyronine (T3) in other vertebrates (Figure 1). Compounds with high structural resemblance with thyroid hormone (especially halogenated phenols, such as hydroxylated metabolites of PCBs or PBDEs), are capable to compete with thyroid hormone for TTR binding. Apart from the fact that this enhances distribution of the toxic compounds, this also causes an increase of unbound thyroid hormone in the blood, which is then freely available for uptake in the liver, metabolic conjugation, and urinary excretion. Ultimately, this may lead to decreased thyroid hormone levels in the blood.

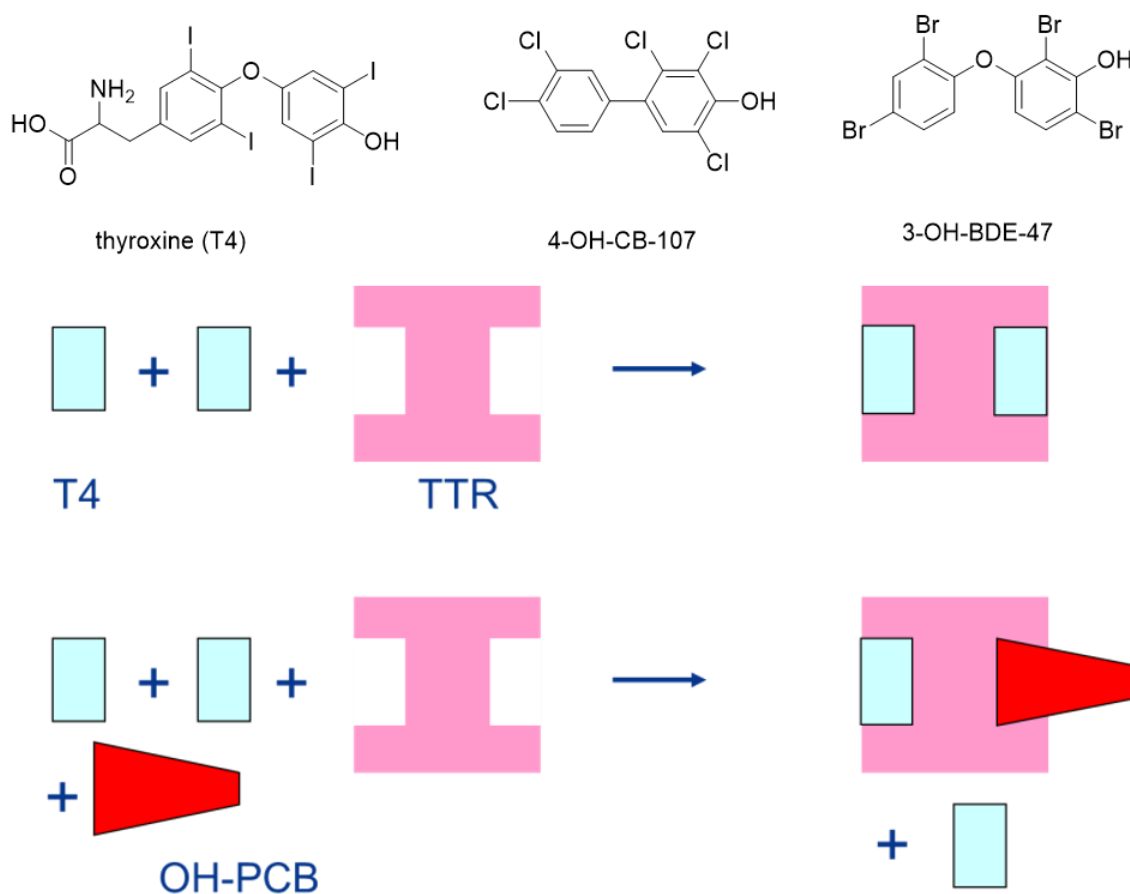
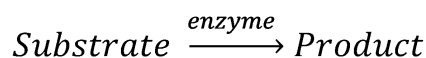


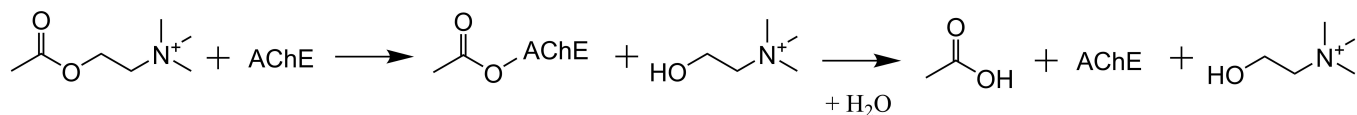
Figure 1: Structural resemblance between T4, a hydroxylated PCB metabolite (4-OH-CB-107) and a hydroxylated PBDE metabolite (3-OH-BDE-47). The lower panel illustrates how halogenated phenols (red; e.g. OH-PCB), given their structural resemblance with T4 (cyan) for TTR-binding (pink), thereby increasing the levels of unbound T4.

## Inhibition of the protein enzymatic activity

Proteins involved in the catalysis of a metabolic reaction are called enzymes. The general formula of such a reaction is



Binding of a toxic compound to an enzyme usually causes an inhibition of the enzyme activity, i.e. a decrease in the conversion rate of the endogenous substrate(s) of the enzyme into its/their corresponding product(s). In practice, this causes a toxic response due to a surplus of substrate and/or a deficit of product. One of the classical examples of enzyme inhibition by toxic compounds is the inhibition of the enzyme acetylcholinesterase (AChE) by organophosphate insecticides. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh), in the cholinergic synapses. During transfer of an action potential from one cell to the other, ACh is released in these synapses from the presynaptic cell into the synaptic cleft in order to stimulate the acetylcholine-receptor (AChR) on the membrane of the postsynaptic cell. AChE, which is also present in these synapses, is then responsible to break down the ACh into acetic acid and choline:



By covalent binding to serine residues in the active site of the AChE enzyme, organophosphate insecticides can inhibit this reaction causing accumulation of the ACh neurotransmitter in the synapse (Fig. 2). As a consequence, the AChR is overstimulated causing convulsions, hypertension, muscle weakness, salivation, lacrimation, gastrointestinal problems, and slow heartbeat.

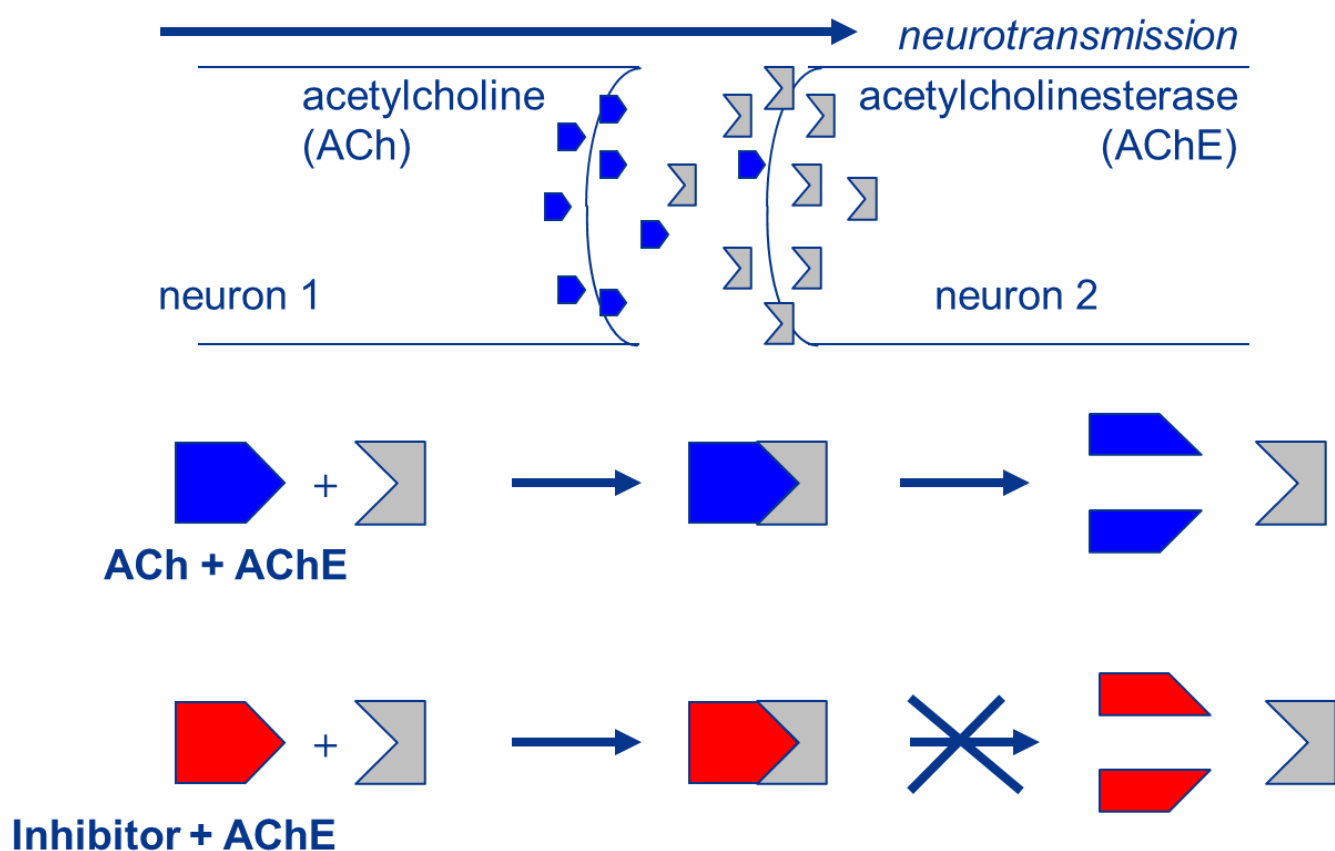


Figure 2: ACh (blue) is released from the presynaptic neuron into the synapse where it merges to and activates the AChR present on membrane of the postsynaptic cell (not shown). Meanwhile, AChE (grey) present in the synaptic cleft hydrolyses the ACh neurotransmitter to avoid overstimulation of the postsynaptic membrane. Organophosphate insecticides (red) bind to the AChE and prevent its reaction with ACh, causing accumulation of ACh.

### Irreversible vs reversible enzyme inhibition

Organophosphate insecticides bind covalently to the AChE enzyme thereby causing irreversible enzyme inhibition. Irreversible enzyme inhibition progressively increases in time following first-order kinetics (link to section on [Bioaccumulation and kinetic modelling](#)). Recovery of enzyme activity can only be obtained by de novo synthesis of enzymes. In contrast to AChE inhibition, inhibition of the T4 transport function of TTR is reversible because the halogenated phenols bind to TTR in a non-covalent way. Similarly, non-covalent binding of a toxic compound to an enzyme causes reversible inhibition of the enzyme activity.



In addition to covalent and non-covalent enzyme binding, irreversible enzyme inhibition may occur when toxic compounds cause an error during enzyme synthesis. For instance, ions of essential metals, which are present as cofactors in the active site of many enzymes, may be replaced by ions of other metals during enzyme synthesis, yielding inactive enzymes. A classic example of such decreased enzyme activity is the inhibition of  $\delta$ -aminolevulinic acid dehydratase ( $\delta$ -ALAD) by lead. In this case, lead replaces zinc in the active site of the enzyme, thereby inhibiting a catalytic step in the synthesis of a precursor of heme, a cofactor of the protein hemoglobin (link to section on [Toxicity mechanisms of metals](#)).

With respect to reversible enzyme inhibition, three types of inhibition can be distinguished, i.e. competitive, non-competitive, and uncompetitive inhibition (Figure 3).

## Enzyme Inhibition (Mechanism)




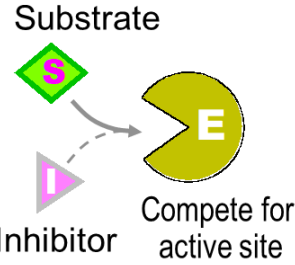
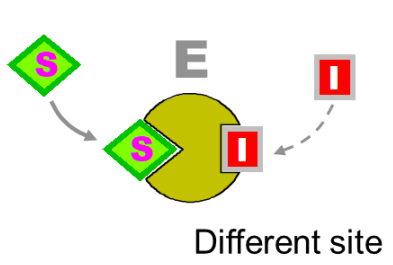
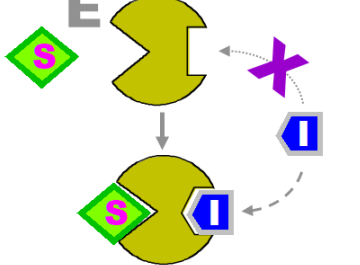
	 Competitive	 Non-competitive	 Uncompetitive
Cartoon Guide	 <p>Substrate</p> <p>Inhibitor</p> <p>Compete for active site</p>	 <p>Different site</p>	
Equation and Description	$E + S \rightleftharpoons ES \rightarrow E + P$ $+ I \rightleftharpoons EI$ <p>[I] binds to free [E] only, and competes with [S]; increasing [S] overcomes Inhibition by [I].</p>	$E + S \rightleftharpoons ES \rightarrow E + P$ $+ I \rightleftharpoons EI + S \rightleftharpoons EIS$ <p>[I] binds to free [E] or [ES] complex; Increasing [S] can not overcome [I] inhibition.</p>	$E + S \rightleftharpoons ES \rightarrow E + P$ $+ I \rightleftharpoons ESI$ <p>[I] binds to [ES] complex only, increasing [S] favors the inhibition by [I].</p>

Figure 3: Three types of reversible enzyme inhibition, i.e. competitive (left), non-competitive (middle), and uncompetitive (right) binding. See text for further explanation. Source: [juang.bst.ntu.edu.tw/files/Enz04%20inhibition.PPT](http://juang.bst.ntu.edu.tw/files/Enz04%20inhibition.PPT)

*Competitive inhibition* refers to a situation where the chemical competes ("fights") with the substrate for binding to the active site of the enzyme. Competitive inhibition is very specific, because it requires that the inhibitor resembles the substrate and fits in the same binding pocket of the active site. The TTR-binding example described above is a typical example of competitive inhibition between thyroid hormone and halogenated phenols for occupation of the TTR-binding site. A more classic example of competitive inhibition is the inhibition of beta-lactamase by penicillin. Beta-lactamase is an enzyme responsible for the hydrolysis of beta-lactam, which is the final step in bacterial cell wall synthesis. By defective cell wall synthesis, penicillin is an antibiotic causing bacterial death.

*Non-competitive inhibition* refers to a situation where the chemical binds to an allosteric site of the enzyme (i.e. not the active site), thereby causing a conformational change of the active site. As a consequence, the substrate cannot enter the active site, or the active site becomes inactive, or the product cannot be released from the active site. For instance, echinocandin antifungal drugs non-competitively inhibit the enzyme 1,3-beta glucan synthase, which is responsible for the synthesis of beta-glucan, a major constituent of the fungal cell wall. Lack of beta-glucan in fungal cell walls prevents fungal resistance against osmotic forces, leading to cell lysis.

*Uncompetitive inhibition* refers to a situation where the chemical can only bind to the enzyme if the substrate is simultaneously bound. Substrate binding leads to a conformational change of the enzyme, which leads to the formation of an allosteric binding site for the inhibitor. Uncompetitive inhibition is more common in two-substrate enzyme reactions than in one-substrate enzyme reactions. An example of uncompetitive inhibition is the inhibition by lithium of the enzyme inositol mono phosphatase (IMPase), which is involved in recycling of the second messenger inositol-3-phosphate (I3P) (link to section on [Receptor interaction](#)). IMPase is involved in the final step of dephosphorylating inositol monophosphate into inositol. Since lithium is the primary treatment for bipolar disorder, this observation has led to the inositol depletion hypothesis that inhibition of inositol phosphate metabolism offers a plausible explanation for the therapeutic effects of lithium.

#### 4.2.1. Question 1

Explain how binding of organophosphate insecticides to acetylcholinesterase enzymes may cause neurotoxicity.

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#### 4.2.1. Question 2

Explain how organohalogenated phenols may cause decreased blood levels of thyroid hormone T4.

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#### 4.2.1. Question 3

What is the difference between a competitive and a non-competitive enzyme inhibitor?

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#### 4.2.1. Question 4

Is it possible to outcompete a competitive enzyme inhibitor by increasing the substrate concentration?

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#### 4.2.1. Question 5

Is it possible to outcompete a non-competitive enzyme inhibitor by increasing the substrate concentration?

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### 4.2.2. Receptor interaction

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#### Learning objectives

You should be able to

- explain the possible effects of compound interference with ion channels.
- explain the possible effects of compound interference with G-protein coupled receptors (GPCRs).
- explain the possible effects of compound interference with enzyme linked receptors.
- explain the possible effects of compound interference with nuclear receptors.
- understand what signalling pathways are and how they can be affected to toxic compounds

**Key words:** Ion channels, G-protein coupled receptors, enzyme linked receptors, nuclear receptors

#### Introduction

Receptor proteins specifically bind and respond to endogenous signalling ligands such as hormones, prostaglandins, growth factors, or neurotransmitters, by causing a typical cellular response. Receptor proteins can be located in the cell membrane, in the cytosol, and in the nucleus of a cell. Agonistic receptor ligands activate the receptor protein whereas antagonistic ligands inactivate the receptor and prevent (endogenous) agonists from activating the receptor (Figure 1). Based on the role of the receptor protein, binding by ligands may interfere with:

1. ion channels
2. G-protein coupled receptors
3. enzyme-linked receptors
4. nuclear receptors.

Xenobiotic ligands can interfere with these cellular responses by acting as agonistic or antagonistic ligands.

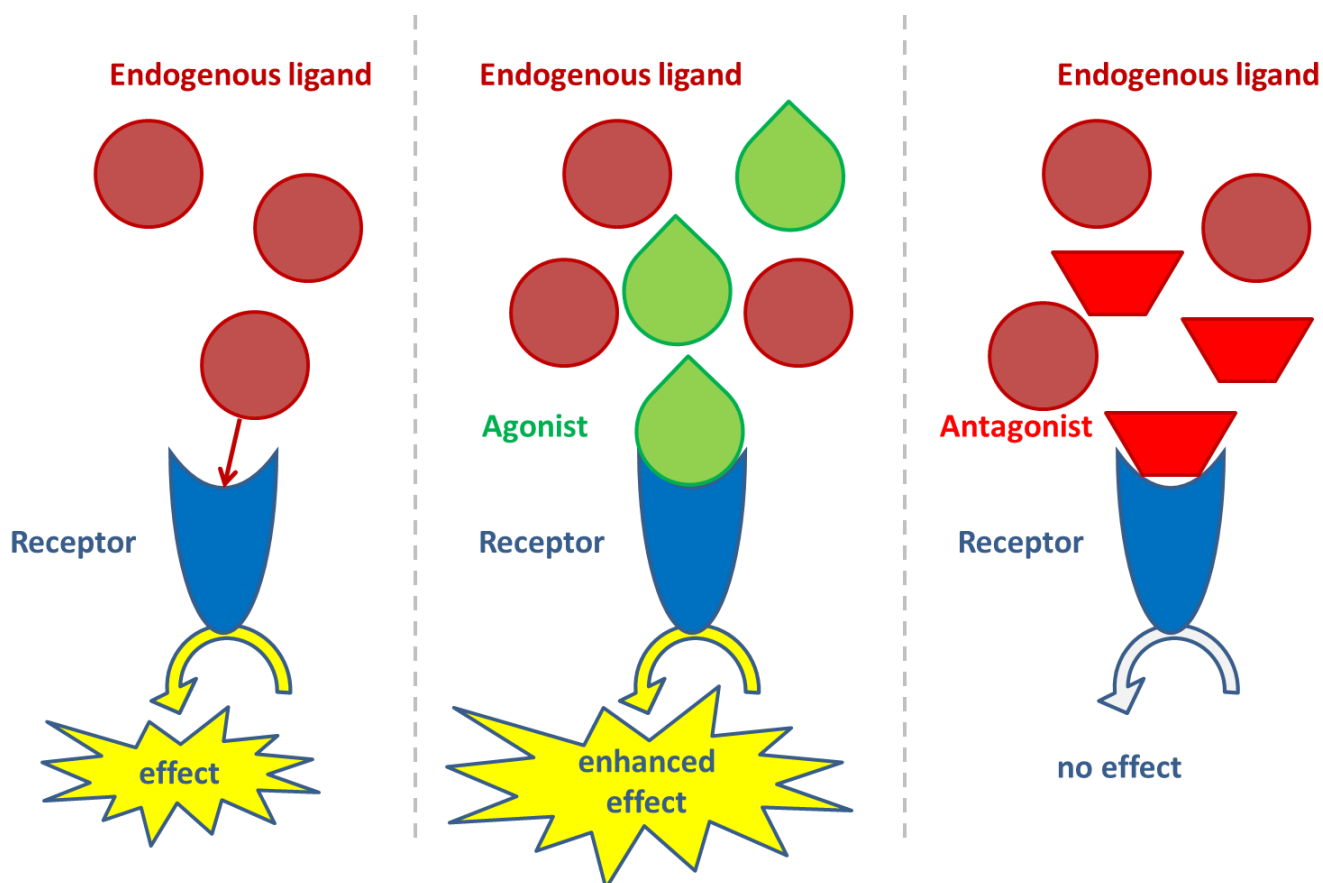


Figure 1: Activation by the endogenous ligand of a receptor leads to an effect. An agonistic compound may also activate the receptor and leads in cooperation with the endogenous ligand to an enhanced effect. An antagonistic compound also has binding affinity for the receptor, but cannot activate it. Instead, it prevents the endogenous ligand from binding, and activating the receptor, thereby preventing the effect.

## 1. Ion channels

Ion channels are transmembrane protein complexes that transport ions across a phospholipid bilayer membrane. Ion channels are especially important in neurotransmission, when stimulating neurotransmitters (e.g. acetylcholine or ACh) bind to the (so-called ionotropic) receptor part of the ion channel and open the ion channel for a very short (i.e. millisecond) period of time. As a result, ions can cross the membrane causing a change in transmembrane potential (see [Figure](#)). On the other hand, receptor-binding by inhibiting neurotransmitters (e.g. gamma-aminobutyric acid or GABA) prevents the opening of ion channels.

Compounds interfering with sodium channels, for instance, are neurotoxic compounds (see section on [Neurotoxicity](#)). They can either block the ion channels or keep them in a prolonged or permanently open state. Many compounds known to interfere with ion channels are natural toxins. For instance, tetrodotoxin (TTX), which is produced by marine bacteria and highly accumulated in puffer fish, and saxitoxin, which is produced by dinoflagellates and is accumulated in shellfish are capable of blocking voltage-gated sodium channels in nerve cells. In contrast, ciguatoxin, which is another persistent toxin produced by dinoflagellates that accumulates in predatory fish positioned high in the food chain, causes prolongation of the opening of voltage-gated sodium channels. Some pesticides like DDT and pyrethroid insecticides also prevent closure of voltage-gated sodium channels in nerve cells. As a consequence, full repolarization of the membrane potential is not achieved. As a consequence, the nerve cells do not reach the resting potential and any new stimulus that would be too low to reach the threshold for depolarization under normal conditions, will now cause a new action potential. In other words, the nerve cells become hyperexcitable and undergo a series of action potentials (repetitive firing) causing tremors and hyperthermia.

## 2. G-protein coupled receptors (GPCRs)

GPCRs are transmembrane receptors that transfer an extracellular signal into an activated G-protein that is connected to the receptor on the intracellular side of the membrane. G-proteins are heterotrimer proteins consisting of three subunits alpha, beta, and

gamma, of which the alpha subunit - in inactivated form - contains a guanosine diphosphate (GDP) molecule. Upon binding by endogenous ligands such as hormones, prostaglandins, or neurotransmitters (i.e. the signal or "first messenger") to the (so-called metabotropic) receptor, a conformational change in the GPCR complex leads to an exchange of the GDP for a guanosine triphosphate (GTP) molecule in the alpha monomer part of the G-protein, causing release of the activated alpha subunit from the beta/gamma dimer part. The activated alpha monomer can interact with several target enzymes causing an increase in "second messengers" starting signal transduction pathways (see point 3 Enzyme-linked receptors). The remaining beta-gamma complex may also move along the inner membrane surface and affect the activity of other proteins (Figure 2).

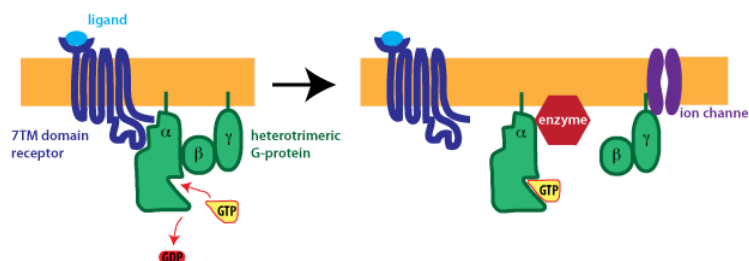


Figure 2: Mechanism of GPCR-activation: ligand binding causes a conformational change leading to the release of an activated alpha monomer, which interacts with a target enzyme (causing an increase of second messengers), and a beta-gamma dimer, which may directly affect activity of other proteins (e.g. an ion channel). Source: [courses.washington.edu/conj/bess/gpcr/gpcr.htm](https://courses.washington.edu/conj/bess/gpcr/gpcr.htm)

Two major enzymes that are activated by the alpha monomer are adenylyl cyclase causing an increase in second messenger cyclic AMP (cAMP) and phospholipase C causing an increase in second messenger diacylglycerol (DAG). In turn, cAMP and DAG activate protein kinases, which can phosphorylate many other enzymes. Activated phospholipase C also causes an increase in levels of the second messenger inositol-3-phosphate (I3P), which opens ion channels in the endoplasmic reticulum causing a release of calcium from the endoplasmic store, which also acts as a second messenger. On the other hand, the increase in cytosolic calcium levels is simultaneously tempered by the beta/gamma dimer, which can inhibit voltage-gated calcium channels in the cell membrane. Ultimately, the GPCR signal is extinguished by slow dephosphorylation of GTP into GDP by the activated alpha monomer, causing it to rearrange with the beta/gamma dimer into the original inactivated trimer G-protein (see also [courses.washington.edu/conj/bess/gpcr/gpcr.htm](https://courses.washington.edu/conj/bess/gpcr/gpcr.htm)).

The most well-known example of disruption of GPCR signalling is by cholera toxin (see text block Cholera toxin below).

Despite the recognized importance of GPCRs in medicine and pharmacology, little attention has so-far been paid in toxicology to interaction of xenobiotics with GPCRs. Although a limited number of studies have demonstrated that endocrine disrupting compounds including PAHs, dioxins, phthalates, bisphenol-A, and DDT can interact with GPCR signalling, the toxicological implications of these interactions (especially with respect to disturbed energetic metabolism) remain subject for further research (see review by Le Ferrec and Øvrevik, 2018).

### Cholera toxin

Cholera toxin is a so-called AB exotoxin by *Vibrio cholerae* bacteria, consisting of an "active" A-part and a "binding" B-part (see <http://www.sumanasinc.com/webcontent/animations/content/diphtheria.html>). Upon binding by the B-part to the intestinal epithelium membrane, the entire AB complex is internalized into the cell via endocytosis, and the active A-part is released. This A-part adds an ADP-ribose group to G-proteins making the GTP dephosphorylation of activated G-proteins impossible. As a consequence, activated G-proteins remain in a permanent active state, adenylyl cyclase is permanently activated and cAMP levels rise, which in turn cause an imbalance in ion housekeeping, i.e. an excessive secretion of chloride ions to the gut lumen and a decreased uptake of sodium ions from the gut lumen. Due to the increased osmotic pressure, water is released to the gut lumen causing dehydration and severe diarrhoea ("rice-water stool").

## 3. Enzyme-linked receptors

Enzyme-linked receptors are transmembrane receptors that transfer an extracellular signal into an intracellular enzymatic activity. Most enzyme-linked receptors belong to the family of receptor tyrosine kinase (RTK) proteins. Upon binding by endogenous ligands such as hormones, cytokines, or growth factors (i.e. the signal or primary messenger) to the extracellular domain of the receptors, the receptor monomers dimerize and develop kinase activity, i.e. become capable of coupling of a phosphate group donated by a high-energy donor molecule to an acceptor protein. The first substrate for this phosphorylation activity is the dimerized receptor itself, which accepts a phosphate group donated by ATP on its intracellular tyrosine residues. This

autophosphorylation is the first step of a signalling pathway consisting of a cascade of subsequent phosphorylation steps of other kinase proteins (i.e. signal transduction), ultimately leading to transcriptional activation of genes followed by a cellular response (Figure 3).

Figure in preparation

**Figure 3:** Upon ligand binding, tyrosine kinase receptor (TKR) proteins become autophosphorylated and may phosphorylate (i.e. activate other proteins), including other kinases.

Xenobiotic compounds can interfere with these signalling pathways in many different ways. Compounds may avoid binding of the endogenous ligand, by blocking the receptor or by chelating the endogenous ligands. Most RTK inhibitors inhibit the kinase activity directly by acting as a competitive inhibitor for ATP binding to the tyrosine residues. Many RTK inhibitors are used in cancer treatment, because RTK overactivity is typical for many types of cancer. This overactivity may for instance be caused by increased levels of receptor-activating growth factors, or to spontaneous dimerization when the receptor is overexpressed or mutated).

#### 4. Nuclear receptors

Nuclear receptors are proteins that are activated by endogenous compounds (often hormones) leading ultimately to expression of genes specifically regulated by these receptors. Apart from ligand binding, activation of most nuclear receptors requires dimerization with a coactivating transcription factor. While some nuclear receptors are located in the nucleus in inactive form (e.g. the thyroid hormone receptor), most nuclear receptors are located in the cytosol, where they are bound to co-repressor proteins (often heat-shock proteins) keeping them in an inactive state. Upon ligand binding to the ligand binding domain (LBD) of the receptor, the co-repressor proteins are released and the receptor either forms a homodimer with a similar activated nuclear receptor or forms a heterodimer with a different nuclear receptor, which is often the retinoid-X receptor (RXR) for nuclear hormone receptors. Before or after dimerization, activated nuclear receptors are translocated to the nucleus. In the nucleus, they bind through their DNA-binding domain (DBD, or "zinc finger") to a responsive element in the DNA located in the promotor region of receptor-responsive genes. Consequently, these genes are transcribed to mRNA in the nucleus, which is further translated into proteins in the cell cytoplasm, see Figure 4).

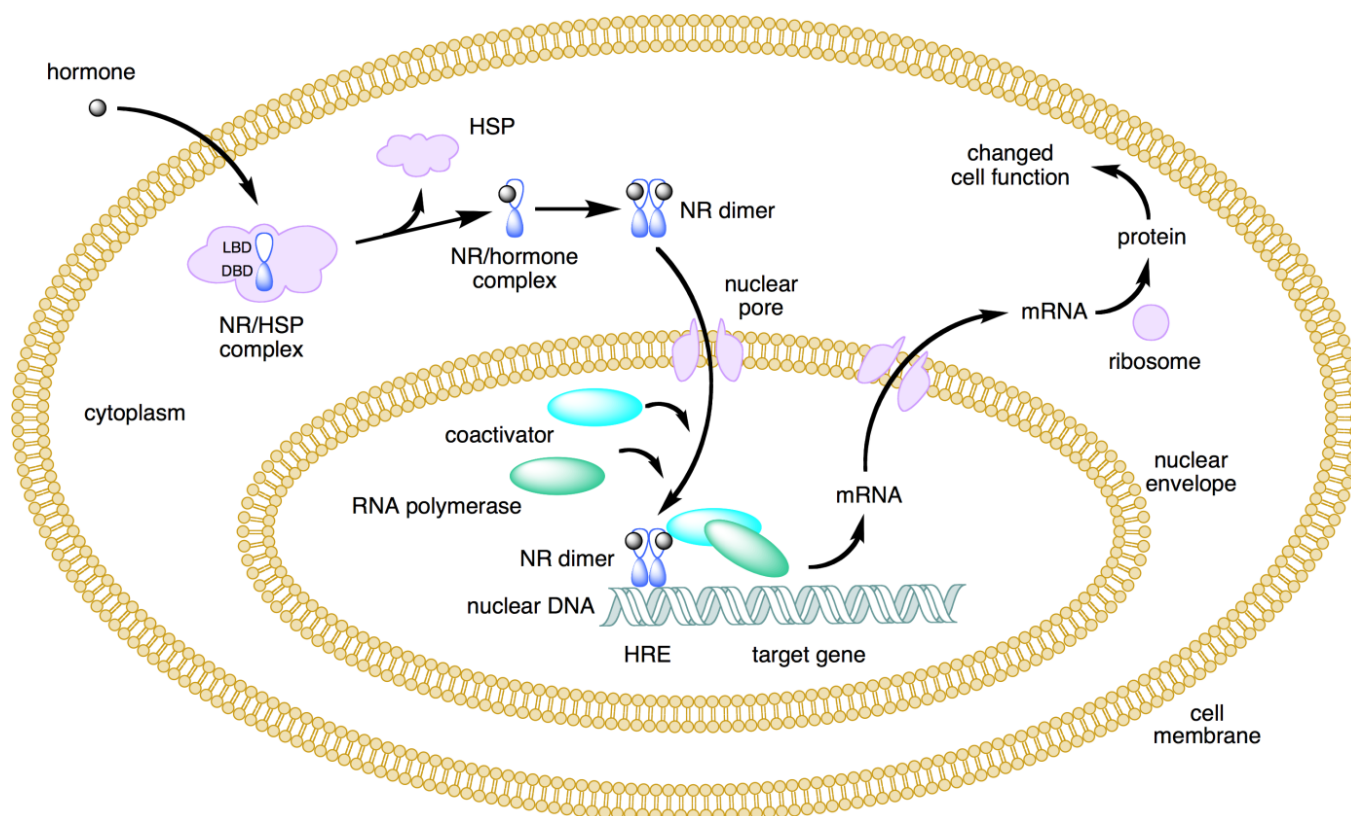
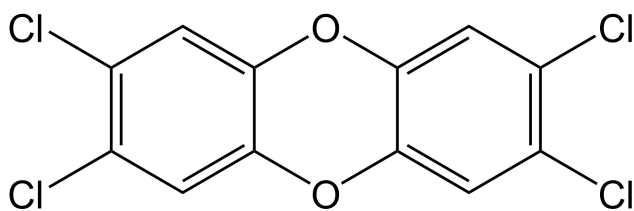


Figure 4: Activation of a cytosolic nuclear receptor (NR). Upon ligand binding (e.g. a hormone), the heat shock proteins (HSP) dissociate from the ligand-receptor complex, which forms a heterodimer before entering the nucleus. After recruiting other coactivating transcription factors, the activated dimer binds to the hormone response element (HRE). RNA polymerase binds to this complex and starts transcription of mRNA, which is excreted from the nucleus into the cytosol and transcribed in corresponding proteins. . Source: [https://upload.wikimedia.org/Wikipedia/commons/3/3f/Nuclear\\_receptor\\_action.png](https://upload.wikimedia.org/Wikipedia/commons/3/3f/Nuclear_receptor_action.png)

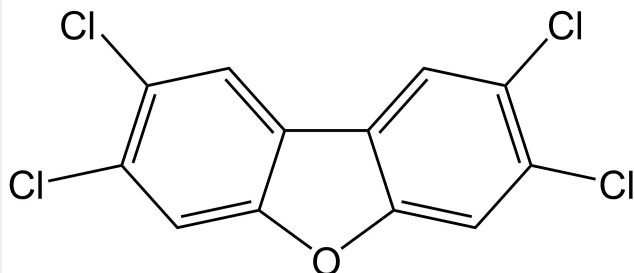
Xenobiotic compounds may act as agonist or antagonists of nuclear receptor activation. Chemicals that act as a nuclear receptor agonist mimic the action of the endogenous activator(s), whereas chemicals that act as a nuclear receptor antagonist basically block the LBD of the receptor, preventing the binding of the endogenous activator(s). Over the past decades, interaction of xenobiotics with nuclear receptors involved in signalling of both steroid and non-steroid hormones has gained a lot of attention of researchers investigating endocrine disruption (link to section on [Endocrine Disruption](#)). Nuclear receptor activation is also the key mechanism in dioxin-like toxicity (see text block dioxin-like toxicity below).

#### Dioxin-like toxicity

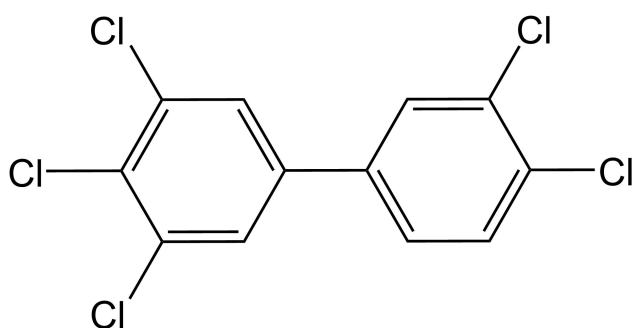
The term dioxins refers to polyhalogenated dibenzo-[p]-dioxin (PHDD) compounds, which are planar molecules consisting of two halogenated aromatic rings, which are connected by two ether bridges. The most potent and well-studied dioxin is 2,3,7,8-tetrachloro-[p]-dibenzodioxin (2,3,7,8-TCDD), which is often too simply referred to as TCDD or even just "dioxin". Other compounds with similar properties (dioxin-like compounds) include polyhalogenated dibenzo-[p]-furan (PHDF) compounds (often too simply referred to as "furans"), which are planar molecules consisting of two halogenated aromatic rings connected by one ether bridge and one carbon-carbon bond. A third major class of dioxin-like compounds belong to the polyhalogenated biphenyls (PHB), which consist of two halogenated aromatic rings connected only by a carbon-carbon bond. The most well-known compounds belonging to this latter category are the polychlorinated biphenyls (PCBs). Of all PHDD, PHDF or PHB compounds, only the persistent and planar compounds are considered dioxin-like compounds. For the PHBs, this implies that they should contain zero or at maximum one halogen-substitution in any of the four ortho-positions (see examples below). Non-ortho-substituted PHBs can easily obtain a planar confirmation with the two aromatic rings in one planar field, whereas mono-ortho-substituted PHBs can obtain such confirmation at higher energetic costs..



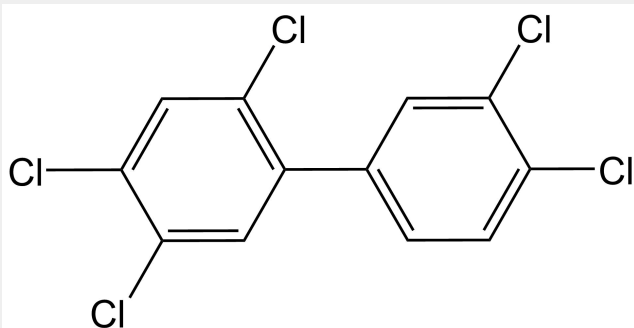
2,3,7,8-tetrachlorodibenzo-[p]-dioxin (2,3,7,8-TCDD) is the most potent and well-studied dioxin-like compound, usually too simply referred to as "dioxin".



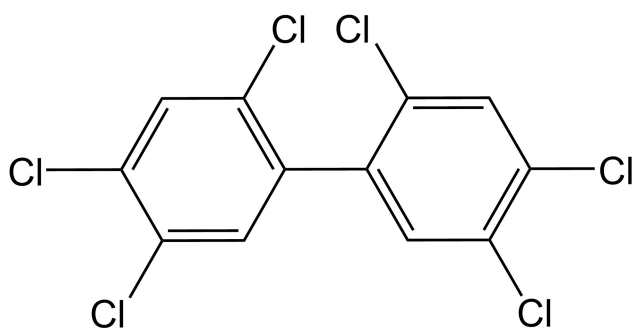
2,3,7,8-tetrachlorodibenzo-[p]-furan (2,3,7,8-TCDF) a dioxin-like compound equally potent to 2,3,7,8-TCDD. It is usually too simply referred to as "furan".



3,3',4,4',5-pentachlorinated biphenyl (PCB-126) is the most potent dioxin-like PCB compound, with no chlorine substitution in any of the four ortho positions next to the carbon-carbon bridge



2,3',4,4',5-pentachlorinated biphenyl (PCB-118) is a weak dioxin-like PCB compound, with one chlorine substitution in the four ortho positions next to the carbon-carbon bridge



2,2',4,4',5,5'-hexachlorinated biphenyl (PCB-153) is a non-dioxin-like (NDL) PCB compound, with two chlorine substitution in the four ortho positions next to the carbon-carbon bridge

The planar composition is required for the dioxin-like compounds to fit as a key in the lock of the arylhydrocarbon (AhR) receptor (also known as the "dioxin-receptor or DR), present in the cytosol. The activated AhR then dissociates from its repressor proteins, is translocated to the nucleus, and forms a heterodimer with the AhR nuclear translocator (ARNT). The AhR-ARNT complex binds to dioxin-response elements (DRE) in the promotor regions of dioxin-responsive genes in the DNA, ultimately leading to transcription and



translation of these genes (see Figure 1 in Denison & Nagy, 2003). Famous examples of such genes belong to the CYP1, UGT, and GST families, which are Phase I and Phase II metabolic enzymes whose activation by the AhR-ARNT complex is a natural response triggered by the need to remove xenobiotics (link to section on [Xenobiotic metabolism and defence](#)). Other genes with a DRE in their promotor region include genes involved in protein phosphorylation, such as the proto-oncogen c-raf and the cyclin dependent kinase inhibitor p27.

This classical mechanism of ligand:AhR:ARNT:DRE complex-dependent induction of gene expression, however, cannot explain all the different types of toxicity observed for dioxins, including immunotoxicity, reproductive toxicity and developmental toxicity. Still, these effects are known to be mediated through the AhR as well, as they were not observed in AhR knockout mice. This can partly be explained by the fact that not all genes that are under transcriptional control of a DRE are known yet. Moreover, AhR dependent mechanisms other than this classical mechanism have been described. For instance, AhR activation may have anti-estrogenic effects because activated AhR (1) binds to the estrogen receptor (ER) and targets it for degradation, (2) binds (with ARNT) to inhibitory DREs in the promotor of ER-dependent genes, and (3) competes with the ER-dimer for common coactivators. Although dioxin-like compounds absolutely require the AhR to exert their major toxicological effects, several AhR independent effects have been described as well, such as AhR-independent alterations in gene expression and changes in  $\text{Ca}^{2+}$  influx related to changes in protein kinase activity. Apart from the persistent halogenated dioxinlike compounds described above, other compounds may also activate the AhR, including natural AhR agonists (nAhRAs) found in food (e.g. indolo[3,2-b]carbazole (ICZ) in cruciferous vegetables, bergamottin in grapefruits, tangeretin in citrus fruits), and other planar aromatic compounds, including polycyclic aromatic hydrocarbons (PAHs) produced by incomplete combustion of organic fuels. Upon activation of the AhR, these non-persistent compounds are metabolized by the induced CYP1A biotransformation enzymes. In addition, an endogenous AhR ligand called 6-formylindolo[3,2-b]carbazole (FICZ) has been identified. FICZ is a mediator in many physiological processes, including immune responses, cell growth and differentiation. Endogenous FICZ levels are regulated by a negative feedback FICZ/AhR/CYP1A loop, i.e. FICZ activates AhR and is metabolized by the subsequently induced CYP1A. Dysregulation of this negative feedback loop by other AhR agonists may disrupt FICZ functioning, and could possibly explain some of the effects observed for dioxinlike compounds.

Further reading:

Denison, M.S., Soshilov, A.A., He, G., De Groot, D.E., Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the Aryl hydrocarbon (Dioxin) Receptor. *Toxicological Sciences* 124, 1-22.

### Further reading:

Boelsterli, U.A. (2009). *Mechanistic Toxicology* (2<sup>nd</sup> edition). Informa Healthcare, New York, London.

Le Ferrec, E., Øvrevik J. (2018). G-protein coupled receptors (GPCR) and environmental exposure. Consequences for cell metabolism using the  $\beta$ -adrenoceptors as example. *Current Opinion in Toxicology* 8, 14-19.

[courses.washington.edu/conj/bess/gpcr/gpcr.htm](https://courses.washington.edu/conj/bess/gpcr/gpcr.htm)

#### 4.2.2. Question 1

Why are compounds interfering with ion channels mainly neurotoxic compounds?

#### 4.2.2. Question 2

GPCR signalling is not only disrupted through interaction with the receptor. What alternative mechanisms can play a role?

#### 4.2.2. Question 3

What is the main effect of activating enzyme-linked receptors?

#### 4.2.2. Question 4

What happens if a compound binds to a nuclear receptor?

### 4.2.3. Oxidative stress - I.

#### Reactive oxygen species and antioxidants

**Author:** Frank van Belleghem



**Reviewers:** Raymond Niesink, Kees van Gestel, Éva Hideg

**Learning objectives:**

You should be able to

- explain what oxidative stress is, under which circumstances it arises and why it is important in toxicology.
- describe what reactive oxygen species are and how they are produced.
- describe how levels of reactive oxygen species are kept under control.
- make a distinction between enzymatic and non-enzymatic antioxidants.

**Keywords:** Reactive oxygen species, Fenton reaction, Enzymatic antioxidants, Non-enzymatic antioxidants, Lipid peroxidation.

## Reactive oxygen species

Molecular oxygen ( $O_2$ ) is a byproduct of photosynthesis and essential to all heterotrophic cells because it functions as the terminal electron acceptor during the oxidation of organic substances in aerobic respiration. This process results in the reduction of  $O_2$  to water, leading to the formation of chemical energy and reducing power. The reason why  $O_2$  can be reduced with relative ease in biological systems can be found in the physicochemical properties of the oxygen molecule (in the triplet ground state, *i.e.* as it occurs in the atmosphere). Because of its electron configuration,  $O_2$  is actually a biradical that can act as an electron acceptor. The outer molecular orbitals of  $O_2$  each contain one electron, the spins of these electrons are parallel (Figure 1). As a result, oxygen (in the ground state) is not very reactive because, according to the Pauli exclusion principle, only one electron at a time can react with other electrons in a covalent bond. As a consequence, oxygen can only undergo univalent reductions, and the complete reduction of oxygen to water requires the sequential addition of four electrons leading to the formation of one-, two-, three-electron oxygen intermediates (Figure 1). These oxygen intermediates are, in sequence, the **superoxide anion radical** ( $O_2^{\bullet-}$ ), **hydrogen peroxide** ( $H_2O_2$ ) and the **hydroxyl radical** ( $\bullet OH$ ).

Another reactive oxygen species of importance is **singlet oxygen** ( $^1O_2$  or  $^1\Delta_g$ ). Singlet oxygen is formed by converting ground-state molecular oxygen into an excited energy state, which is much more reactive than the normal ground-state molecular oxygen. Singlet oxygen is typically generated by a process called photosensitization, for example in the lens of the eye. Photosensitization occurs when light (UV) absorption by an endogenous or xenobiotic substance lifts the compound to a higher energy state (a high-energy triplet intermediate) which can transfer its energy to oxygen, forming highly reactive singlet oxygen. Apart from oxygen-dependent photodynamic reactions, singlet oxygen is also produced by neutrophils and this has been suggested to be important for bacterial killing through the formation of ozone ( $O_3$ ) (Onyango, 2016).

Because these oxygen intermediates are potentially deleterious products that can damage cellular components, they are referred to as **reactive oxygen species (ROS)**. ROS are also often termed 'free radicals' but this is incorrect because not all ROS are radicals (e.g.  $H_2O_2$ ,  $^1O_2$  and  $O_3$ ). Moreover, as all radicals are (currently) considered as unattached, the prefix 'free' is actually unnecessary (Koppenol & Traynham, 1996).

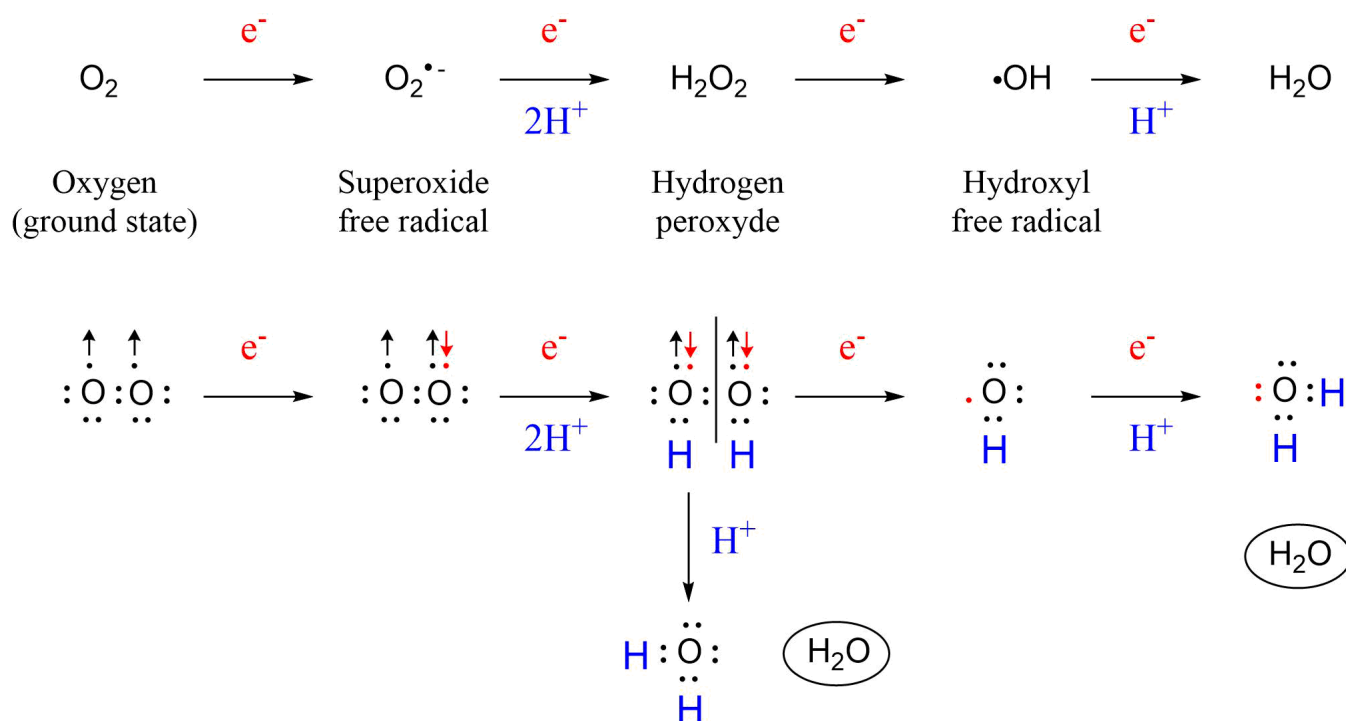


Figure adapted from Edreva (2005) by Steven Droge.

ROS are byproducts of aerobic metabolism in the different organelles of cells, for instance respiration or photosynthesis, or as part of defenses against pathogens. Endogenous sources of reactive oxygen species include oxidative phosphorylation, P450 metabolism, peroxisomes and inflammatory cell activation. For example, superoxide anion radicals are endogenously formed from the reduction of oxygen by the semiquinone of ubiquinone (coenzyme Q), a coenzyme widely distributed in plants, animals, and microorganisms. Ubiquinones function in conjunction with enzymes in cellular respiration (*i.e.*, oxidation-reduction processes). The superoxide anion radical is formed when one electron is taken up by one of the antibonding  $\pi^*$ -orbitals (formed by two 2p atomic orbitals) of molecular oxygen.

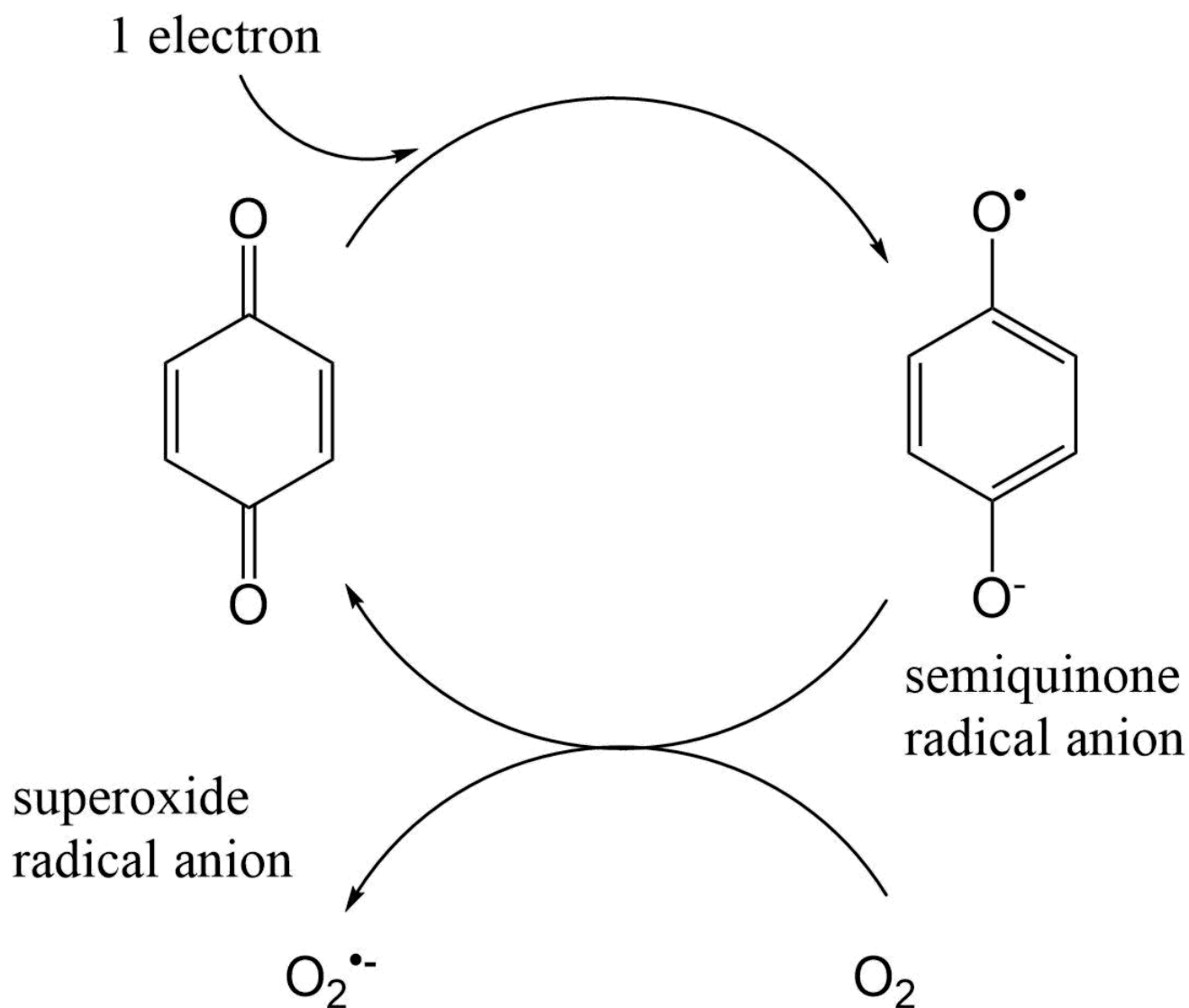


Figure adapted from Bolton & Dunlap (2016) by Steven Droge.

A second example of an endogenous source of superoxide anion radicals is the auto-oxidation of reduced heme proteins. It is known, for example, that oxyferrocycytochrome P-450 substrate complexes may undergo auto-oxidation and subsequently split into (ferri) cytochrome P-450, a superoxide anion radical and the substrate (S). This process is known as the uncoupling of the cytochrome P-450 (CYP) cycle and also referred to as the oxidase activity of cytochrome P-450. However, it should be mentioned that this is not the normal functioning of CYP. Only when the transfer of an oxygen atom to a substrate is not tightly coupled to NADPH utilization, so that electrons derived from NADPH are transferred to oxygen to produce  $O_2^{\bullet -}$  (and also  $H_2O_2$ ).

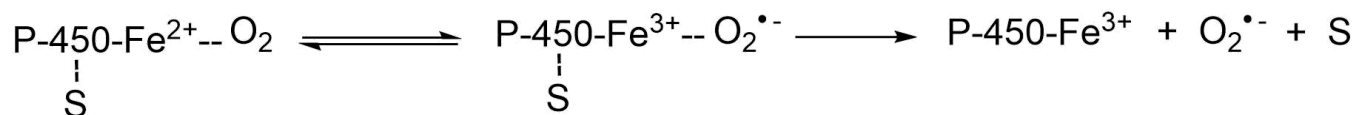


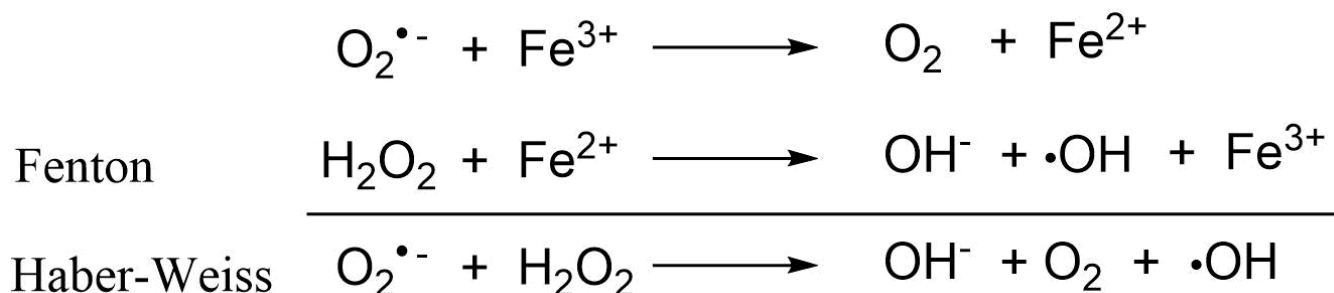
Table 1 shows the key oxygen species and their biological half-life, their migration distance, the endogenous source and their reaction with biological compounds.

**Table 1.** The key oxygen species and their characteristics (table adapted from Das & Roychoudhury, 2014)

ROS species	Half-life ( $T_{1/2}$ )	Migration distance	Endogenous source	Mode of action

Superoxide anion radical ( $O_2^{\bullet-}$ )	1-4 $\mu s$	30 nm	Mitochondria, cytochrome P450, macrophage/inflammatory cells, membranes, chloroplasts	Reacts with compounds with double bonds
Hydroxyl radical ( $\bullet OH$ )	1 $\mu s$	1 nm	Mitochondria, membranes, chloroplasts	Reacts vigorously with all biomolecules.
Hydrogen peroxide ( $H_2O_2$ )	1 ms	1 $\mu m$	Mitochondria, membranes, peroxisomes, chloroplasts	Oxidizes proteins by reacting with the Cys residue.
Singlet Oxygen	1-4 $\mu s$	30 nm	Mitochondria, membranes, chloroplasts	Oxidizes proteins, polyunsaturated fatty acids and DNA

Because of their reactivity, at elevated levels ROS can indiscriminately damage cellular components such as lipids, proteins and nucleic acids. In particular the superoxide anion radical and hydroxyl radicals that possess an unpaired electron are very reactive. In fact, hydroxyl has the highest 1-electron reduction potential, making it the single most reactive radical known. Hydroxyl radicals (Figure 1) can arise from hydrogen peroxide in the presence of redox-active transition metal, notably  $Fe^{2+/3+}$  or  $Cu^{+/2+}$ , via the **Fenton reaction**. In case of iron, for this reaction to take place, the oxidized form ( $Fe^{3+}$ ) has to be reduced to  $Fe^{2+}$ . This means that  $Fe^{2+}$  is only released in an acidic environment (local hypoxia) or in the presence of superoxide anion radicals. The reduction of  $Fe^{3+}$ , followed by the interaction with hydrogen peroxide, leading to the generation of hydroxyl radical, is called the iron catalyzed **Haber-Weiss reaction**.



Deze afbeelding  
is niet (meer)  
beschikbaar.

### Keeping reactive oxygen species under control

In order to keep the ROS concentrations at low physiologic levels, aerobic organisms have evolved complex antioxidant defense systems that include antioxidant components that are enzymatic and non-enzymatic. These are cellular mechanisms that are evolved to inhibit oxidation by quenching ROS. Three classes of **enzymes** are known to provide protection against reactive oxygen species: the **superoxide dismutases** that catalyze the dismutation of the superoxide anion radical, and the **catalases** and **peroxidases** that react specifically with hydrogen peroxide. These antioxidant enzymes can be seen as a **first-line defense** as they prevent the conversion of the less reactive oxygen species, superoxide anion radical and hydrogen peroxide, to more reactive species such as the hydroxyl radical. **The second line of defense** largely consists of **non-enzymatic substances** that eliminate radicals such as **glutathione** and **vitamins E and C**. An overview of the cellular defense system is provided in Figure 3.

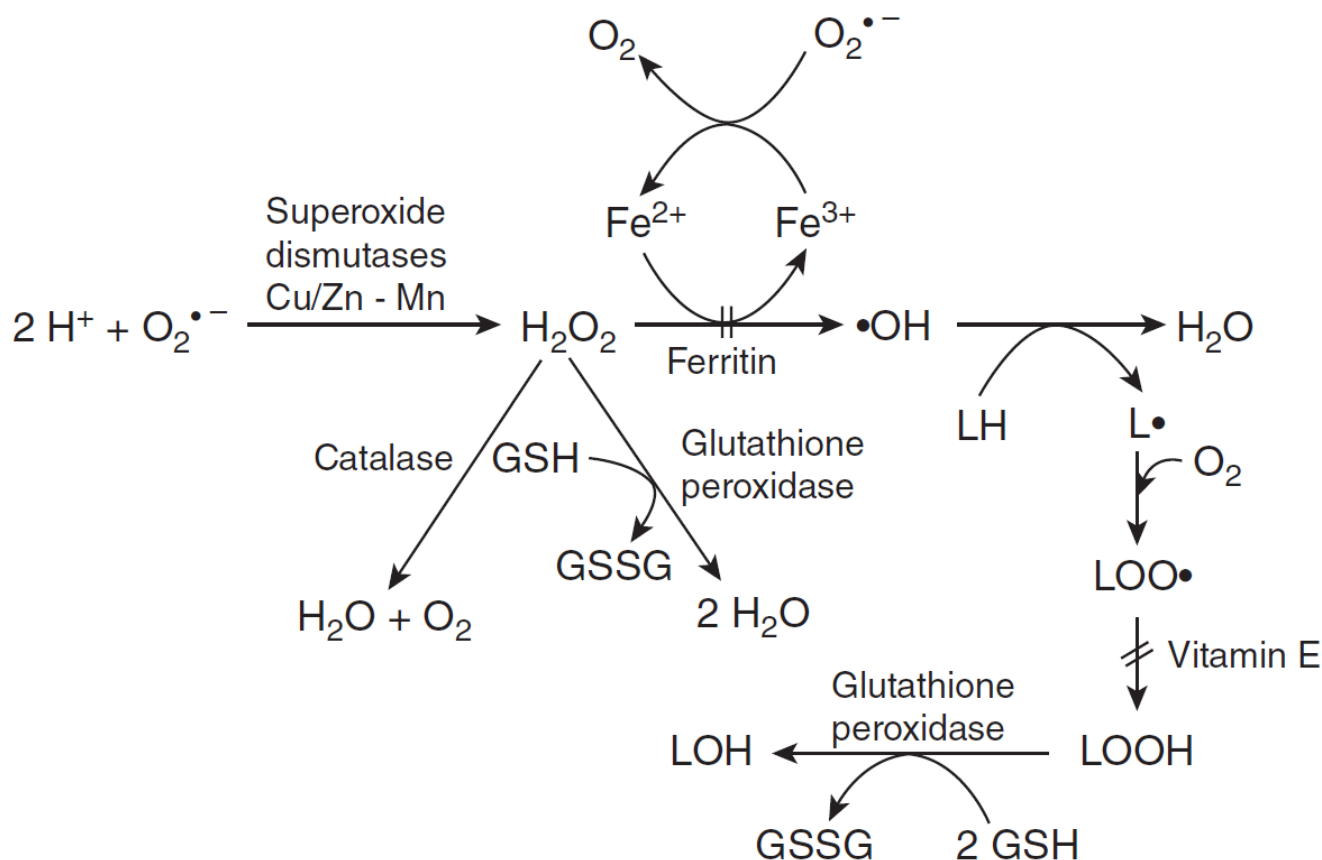
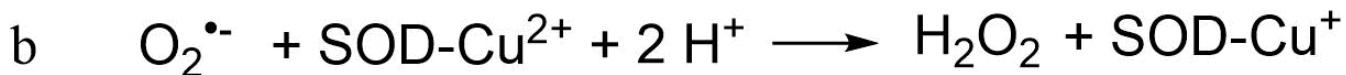
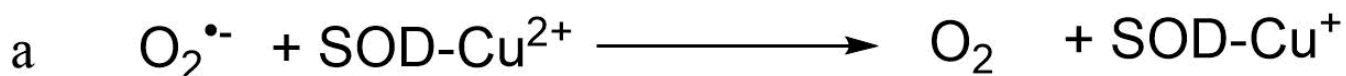


Figure adapted from Smart & Hodgson (2018) by Steven Droge.

## Enzymatic antioxidants

**Superoxide dismutases (SODs)** are metal-containing proteins (metalloenzymes) that catalyze the dismutation of the superoxide anion radical to molecular oxygen in the ground state and hydrogen peroxide, as illustrated by following reactions:



Dismutation of superoxide anion radicals acts in the first part of the reaction with the superoxide anion radical as a reducing agent (a), and as an oxidant in the second part (b). Different types of SOD are located in different cellular locations, for instance Cu-Zn-SOD are mainly located in the cytosol of eukaryotes, Mn-SOD in mitochondria and prokaryotes, Fe-SOD in chloroplasts and prokaryotes and Ni-SOD in prokaryotes. Mn, Fe, Cu and Ni are the redox active metals in the enzymes, whereas Zn not being catalytic in the Cu-Zn-SOD.

$H_2O_2$  is further degraded by catalase and peroxidase. **Catalase (CAT)** contains four iron-containing heme groups that allow the enzyme to react with the hydrogen peroxide and is usually located in peroxisomes, which are organelles with a high rate of ROS production. Catalase converts hydrogen peroxide to water and oxygen. In fact, catalase cooperates with superoxide dismutase in the removal of the hydrogen peroxide resulting from the dismutation reaction. Catalase acts only on hydrogen peroxide, not on organic hydroperoxide.

**Peroxidases (Px)** are hemoproteins that utilize  $H_2O_2$  to oxidize a variety of endogenous and exogenous substrates. An important peroxidase enzyme family is the selenium-cysteine containing **Glutathione peroxidase (GPx)**, present in the cytosol and mitochondria. It catalyzes the conversion of hydrogen  $H_2O_2$  to  $H_2O$  via the oxidation of reduced glutathione (GSH) into its

disulfide form glutathione disulfide (GSSG). Glutathione peroxidase catalyzes not only the conversion of hydrogen peroxide, but also that of organic peroxides. It can transform various peroxides, e.g. the hydroperoxides of lipids. Glutathione peroxidase is found in both the cytosol and in the mitochondria. In the cytosol, the enzyme is present in special vesicles.

Another group of enzymes, not further described here, are the **Peroxiredoxins (Prxs)**, present in the cytosol, mitochondria, and endoplasmic reticulum, use a pair of cysteine residues to reduce and thereby detoxify hydrogen peroxide and other peroxides. It has to be mentioned that no enzymes react with hydroxyl radical or singlet oxygen.

## Non-enzymatic antioxidants

The *second line of defense* largely consists of **non-enzymatic substances** that eliminate radicals. The major antioxidant is **glutathione (GSH)**, which acts as a nucleophilic scavenger of toxic compounds, trapping electrophilic metabolites by forming a thioether bond between the cysteine residue of GSH and the electrophile. The result generally is a less reactive and more water-soluble conjugate that can easily be excreted (see also [phase II biotransformation reactions](#)). GSH also is a co-substrate for the enzymatic (GS peroxidase-catalyzed) degradation of  $H_2O_2$  and it keeps cells in a reduced state and is involved in the regeneration of oxidized proteins.

Other important radical scavengers of the cell are the vitamins E and C. **Vitamin E ( $\alpha$ -tocopherol)** is lipophilic and is incorporated in cell membranes and subcellular organelles (endoplasmic reticulum, mitochondria, cell nuclei) and reacts with lipid peroxides.  $\alpha$ -Tocopherol can be divided into two parts, a lipophilic phytyl tail (intercalating with fatty acid residues of phospholipids) and a more hydrophilic chromanol head with a phenolic group (facing the cytoplasm). This phenolic group can reduce radicals (e.g. lipid peroxy radicals ( $LOO^\bullet$ ), see Figure 2, for explanation of lipid peroxidation, see section on [Oxidative stress II: induction by chemical exposure and possible effects](#)) and is thereby oxidized in turn to the tocopheryl radical which is relatively unreactive because it is stabilized by resonance. The radical is regenerated by vitamin C or by reduced glutathione (Figure 4). Oxidized non-enzymatic antioxidants are regenerated by various enzymes such as glutathione.

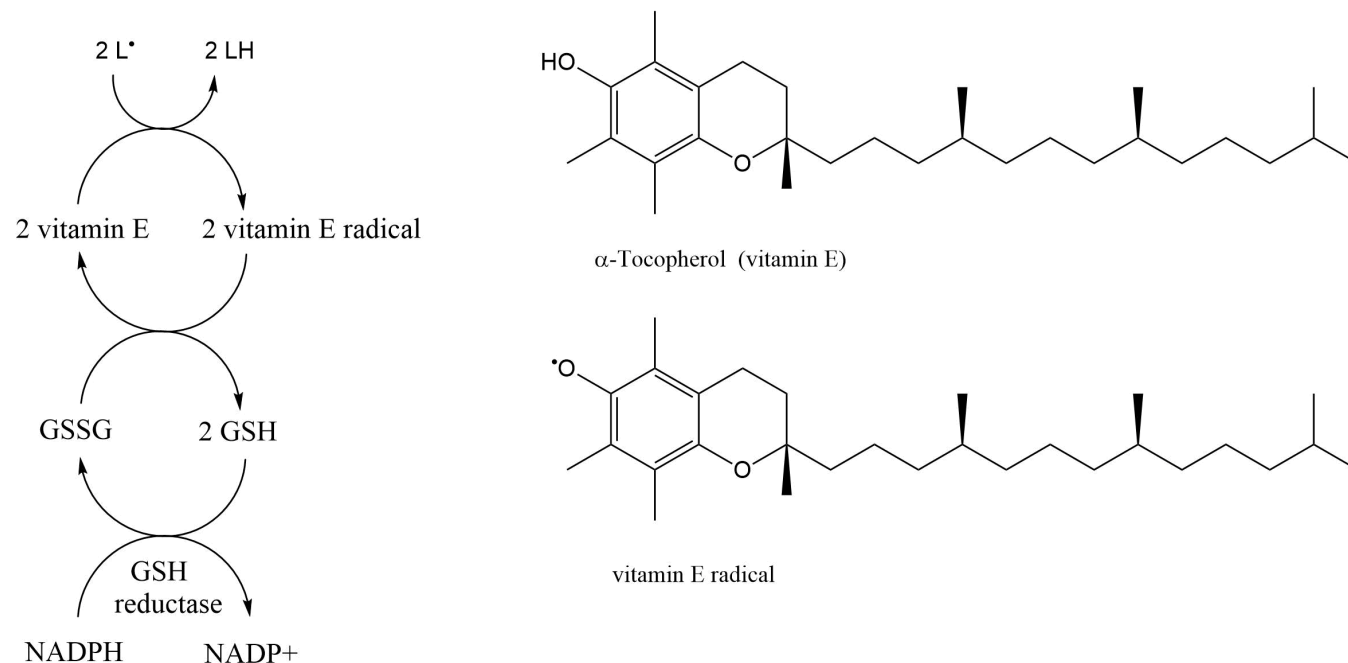


Figure adapted from Niesink et al. (1996) by Steven Droge.

**Vitamin C** (ascorbic acid) is a water-soluble antioxidant and is present in the cytoplasm. Ascorbic acid is an electron donor which reacts quite rapidly with the superoxide anion radical and peroxy radicals, but is generally ineffective in detoxifying hydroxyl radicals because of its extreme reactivity it does not reach the antioxidant (See Klaassen, 2013). Moreover, it regenerates  $\alpha$ -Tocopherol in combination with reduced GSH or compounds capable of donating reducing equivalents (Nimse and Pal, 2015); Figure 5.

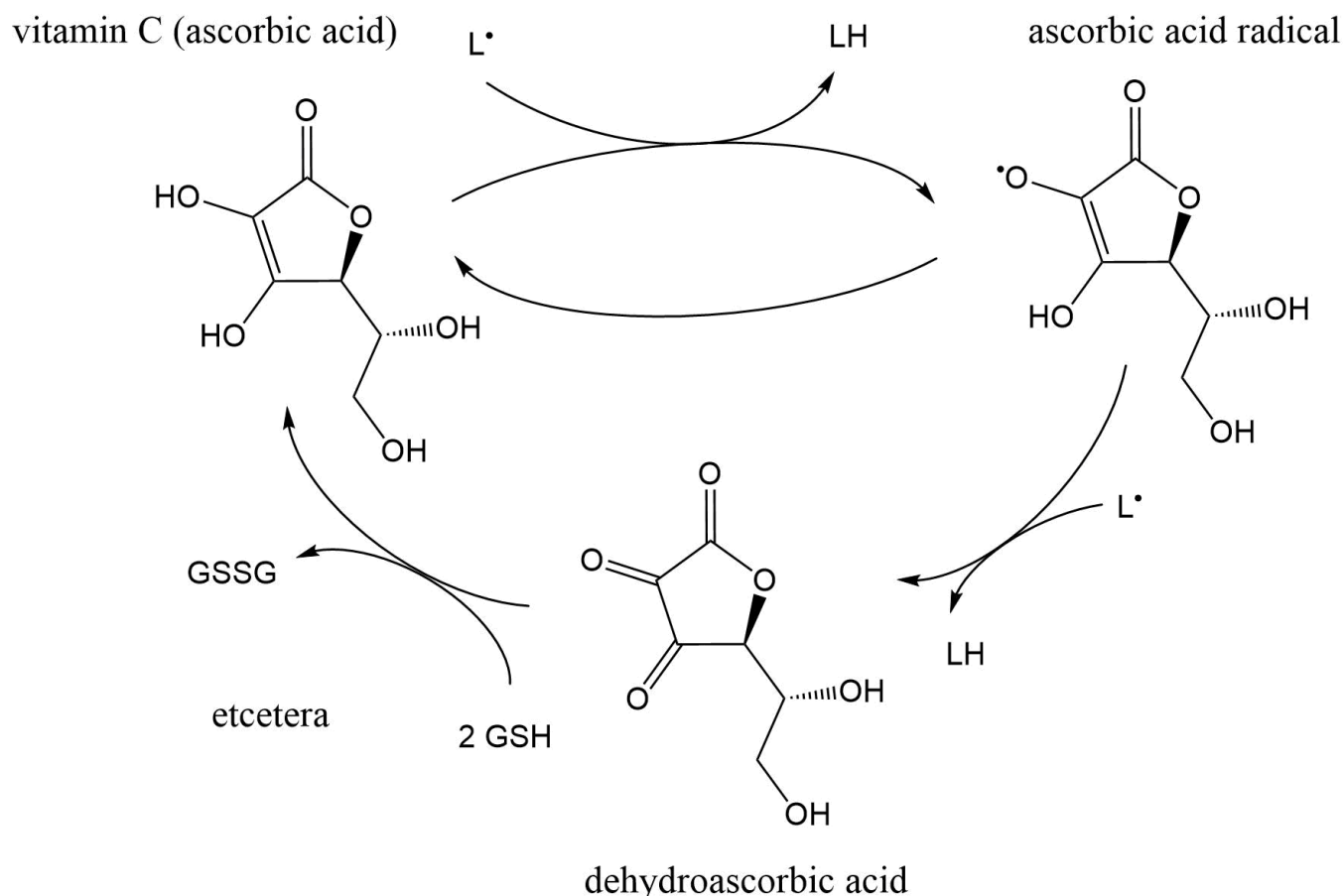


Figure adapted from Niesink et al. (1996) by Steven Droge.

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- Smart, R.C., Hodgson, E. (Eds.). (2018). *Molecular and Biochemical Toxicology*. John Wiley & Sons.

### 4.2.3.I. Question 1

What are the chances of hydroxyl radicals being formed inside the cell? On what factors does such formation depend?



#### 4.2.3.I. Question 2

Given:

Two oxygen species:

I atmospheric oxygen ( $O_2$ )

II singlet oxygen ( $^1O_2$ )

Which oxygen species contains one or more unpaired electrons, and therefore has radical properties?

I and II

only I

only II

neither I nor II

---

#### 4.2.3.I. Question 3

Which of the following radicals are detoxified by  $\alpha$ -tocopherol (vitamine E)?

I hydroxyl radical,  $^{\bullet}OH$

II superoxide anion radical,  $O_2^{\bullet-}$

III lipid radical ( $L^{\bullet}$ )

IV lipid peroxy radical ( $LOO^{\bullet}$ )

I and II

III and IV

I, II and III

II, III and IV

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#### 4.2.3.I. Question 4

Given:

Three enzymes:

I catalase

II peroxidase

III superoxide dismutase

What enzyme removes hydrogen peroxide?

I and II

I and III

II and III

only III

---

#### 4.2.3. Oxidative stress - II.

##### Induction by chemical exposure and possible effects

**Author:** Frank van Bellegheem

**Reviewers:** Raymond Niesink, Kees van Gestel, Éva Hideg

##### Learning objectives:

You should be able to

- explain how xenobiotic compounds can lead to an increased production of.
- explain what oxidative stress does with
  - proteins,
  - lipids,
  - DNA and
  - gene regulation.

**Keywords:** prooxidant-antioxidant balance, bioactivation, oxidative damage,

### How xenobiotic compounds induce generation of ROS

The formation of reactive oxygen species (ROS; see section on [Oxidative stress I](#)) may involve endogenous substances and chemical-physiological processes as well as xenobiotics. Experimental evidence has shown that oxidative stress can be considered as one of the key mechanisms contributing to the cellular damage of many toxicants. **Oxidative stress** has been defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former", leading to potential damage. It is the point at which the production of ROS exceeds the capacity of antioxidants to prevent damage (Klaassen et al., 2013).

Xenobiotics involved in the formation of the superoxide anion radical are mainly substances that can be taken up in so reactive oxygen species -called **redox cycles**. These include quinones and hydroquinones in particular. In the case of quinones the redox cycle starts with a one-electron reduction step, just as in the case of benzoquinone (Figure 1). The resulting benzosemiquinone subsequently passes the electron received on to molecular oxygen. The reduction of quinones is catalyzed by the NADPH-dependent cytochrome P-450 reductase.

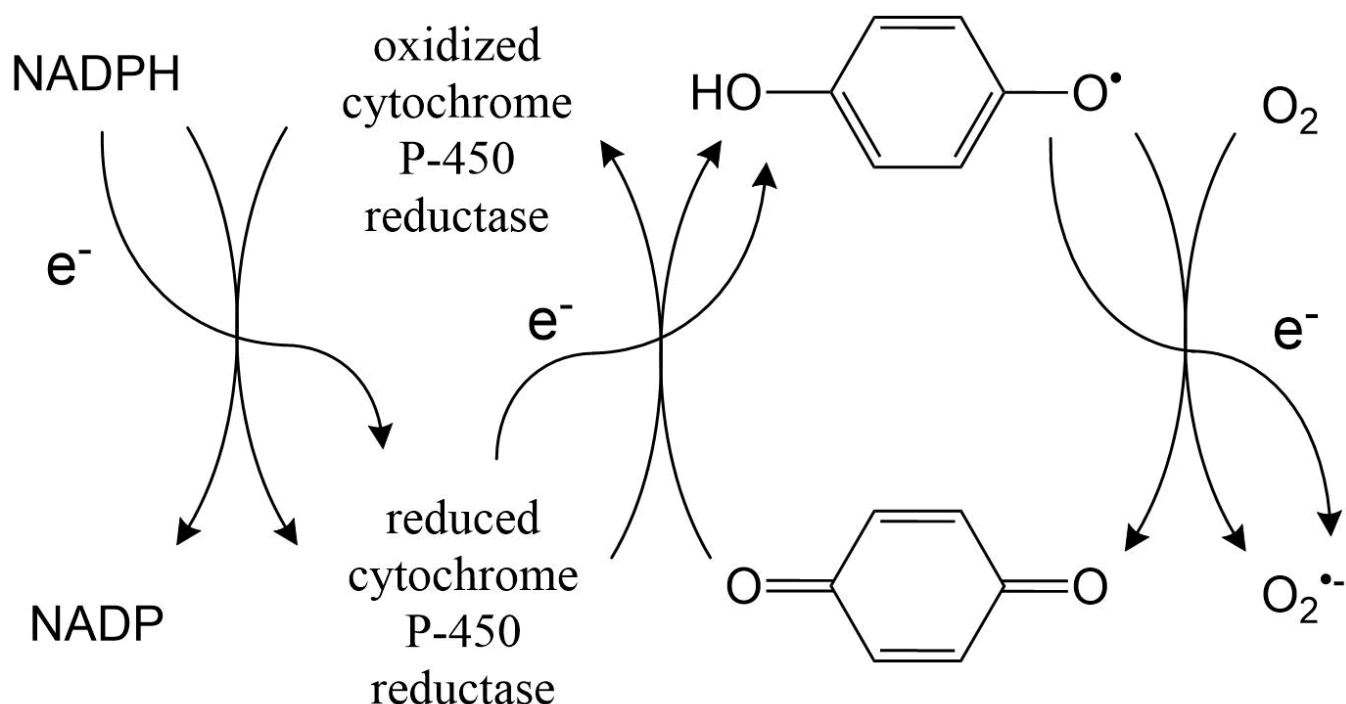


Figure adapted from Niesink et al. (1996) by Steven Droge.

Obviously, hydroquinones can enter a redox cycle via an oxidative step. This step may be catalyzed by enzymes, for example prostaglandin synthase.

Other types of xenobiotic that can be taken up in a redox cycle, are the bipyridyl derivatives. A well-known example is the herbicide paraquat, which causes injury to lung tissue in humans and animals. Figure 2 schematically shows its bioactivation. Other compounds that are taken up in a redox cycle are nitroaromatics, azo compounds, aromatic hydroxylamines and certain metal (particularly Cu and Zn) chelates.

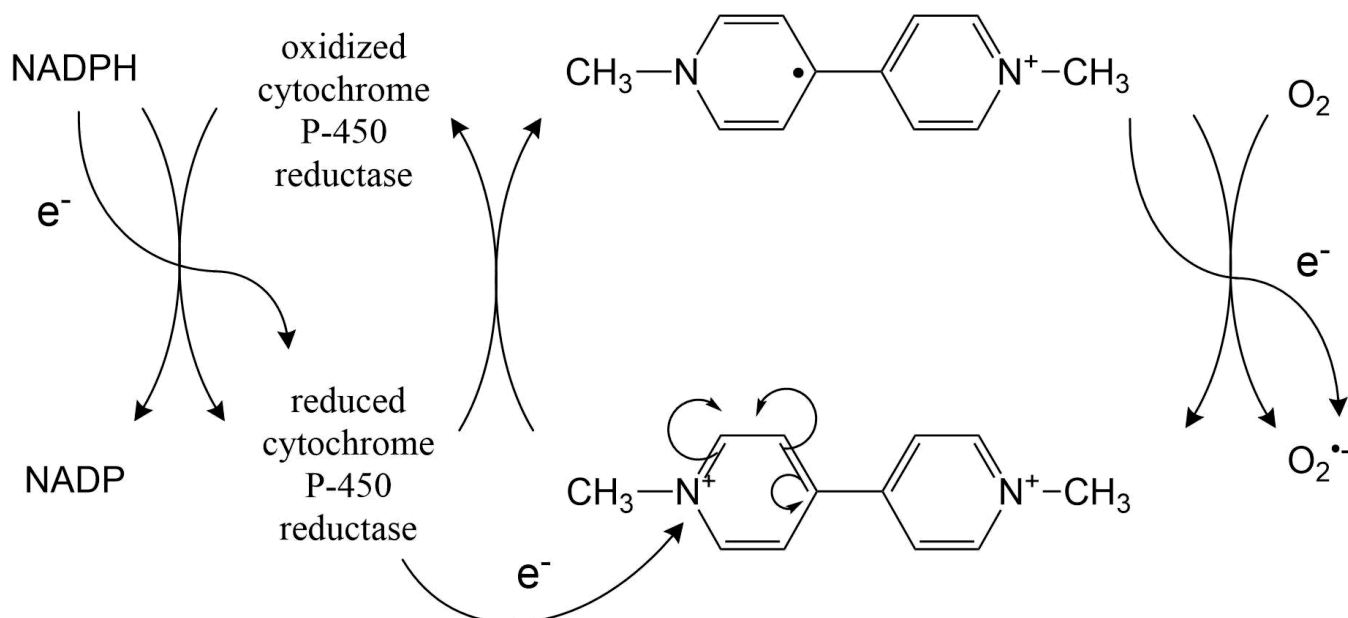


Figure adapted from Niesink et al. (1996) by Steven Droge.

Xenobiotics can enhance ROS production if they are able to enter mitochondria, microsomes, or chloroplasts and **interact with the electron transport chains**, thus blocking the normal electron flow. As a consequence, and especially if the compounds are electron acceptors, they divert the normal electron flow and increase the production of ROS. A typical example is the cytostatic drug doxorubicin, a well-known chemotherapeutic agent, which is used in treatment of a wide variety of cancers. Doxorubicin has a high affinity for cardiolipin, an important compound of the inner mitochondrial membrane and therefore accumulates at that subcellular location.

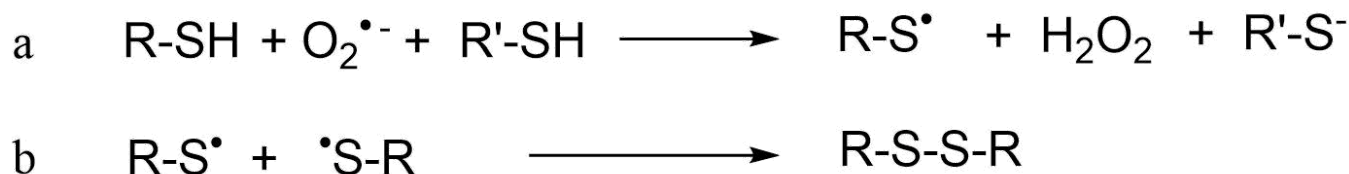
Xenobiotics can cause oxidative damage **indirectly** by interfering with the antioxidative mechanisms. For instance it has been suggested that as a non-Fenton metal, cadmium (Cd) is unable to directly induce ROS. However, indirectly, Cd induces oxidative stress by a displacement of redox-active metals, depletion of redox scavengers (glutathione) and inhibition of antioxidant enzymes (protein bound sulfhydryl groups) (Cuypers et al., 2010; Thévenod et al., 2009).

### The mechanisms of oxidative stress

As mentioned before, oxidative stress has been defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former". ROS can damage proteins, lipids and DNA via direct oxidation, or through redox sensors that transduce signals, which in turn can activate cell-damaging processes like apoptosis.

### Oxidative protein damage

Xenobiotic-induced generation of ROS can damage **proteins** through the oxidation of side chains of amino acids residues, the formation of protein-protein cross-links and fragmentation of proteins due to peptide backbone oxidation. The sulfur-containing amino acids cysteine and methionine are particularly susceptible for oxidation. An example of side chain oxidation is the direct interaction of the superoxide anion radical with sulfhydryl (thiol) groups, thereby forming thiyl radicals as intermediates:



As a consequence, glutathione, composed of three amino acids (cysteine, glycine, and glutamate) and an important cellular reducing agent, can be damaged in this way. This means that if the oxidation cannot be compensated or repaired, oxidative stress can lead to depletion of reducing equivalents, which may have detrimental effects on the cell.

Fortunately, antioxidant defence mechanisms limit the oxidative stress and the cell has repair mechanisms to reverse the damage. For example, heat shock proteins (hsp) are able to renature damaged proteins and oxidatively damaged proteins are degraded by the proteasome.

#### Oxidative lipid damage

Increased concentrations of reactive oxygen radicals can cause membrane damage due to **lipid peroxidation** (oxidation of polyunsaturated lipids). This damage may result in altered membrane fluidity, enzyme activity and membrane permeability and transport characteristics. An important feature characterizing lipid peroxidation is the fact that the initial radical-induced damage at a certain site in a membrane lipid is readily amplified and propagated in a **chain-reaction-like fashion**, thus dispersing the damage across the cellular membrane. Moreover, the products arising from lipid peroxidation (e.g. alkoxy radicals or toxic aldehydes) may be equally reactive as the original ROS themselves and damage cells by additional mechanisms. The chain reaction of lipid peroxidation consists of three steps:

1. Abstraction of a hydrogen atom from a polyunsaturated fatty acid chain by reactive oxygen radicals (*radical formation, initiation*).
2. Reaction of the resulting fatty acid radical with molecular oxygen (*oxygenation* or, more specifically, *peroxidation, propagation*)
3. These events may be followed by a detoxification process, in which the reaction chain is stopped. This process, which may proceed in several steps, is sometimes referred to as *termination*.

Figure 3 summarizes the various stages in lipid peroxidation.

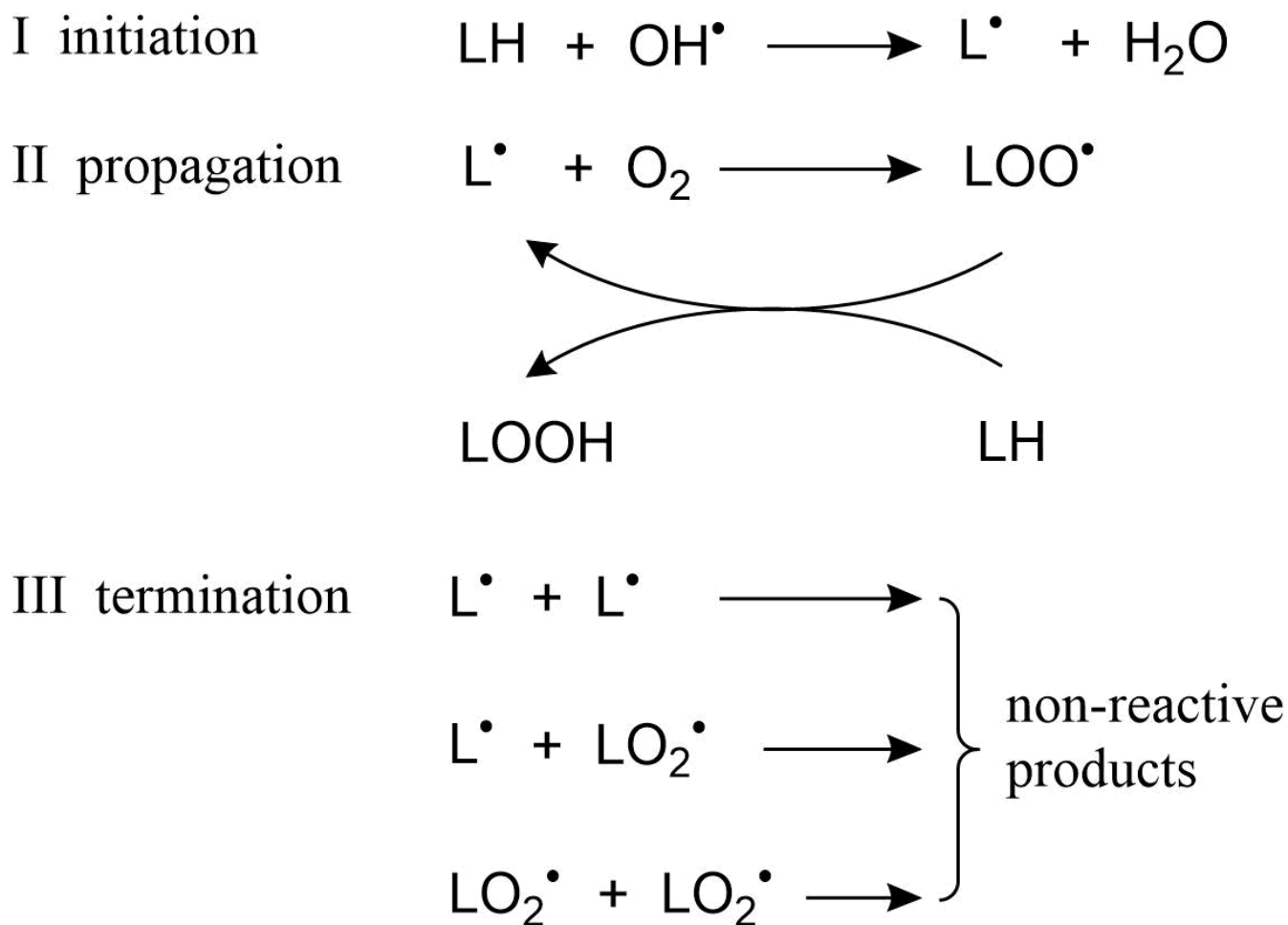


Figure adapted from Niesink et al. (1996) by Steven Droge.

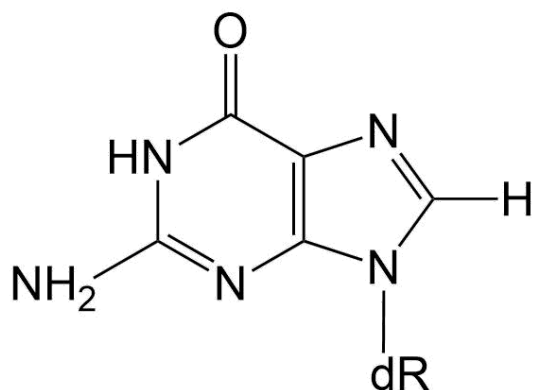
In step II, the peroxidation of biomembranes generates a variety of reactive electrophiles such as epoxides ( $\text{LOO}^\bullet$ ) and aldehydes, including malondialdehyde (MDA). MDA is a highly reactive aldehyde which exhibits reactivity toward nucleophiles and can form

MDA-MDA dimers. Both MDA and the MDA-MDA dimers are mutagenic and indicative of oxidative damage of lipids from a variety of toxicants.

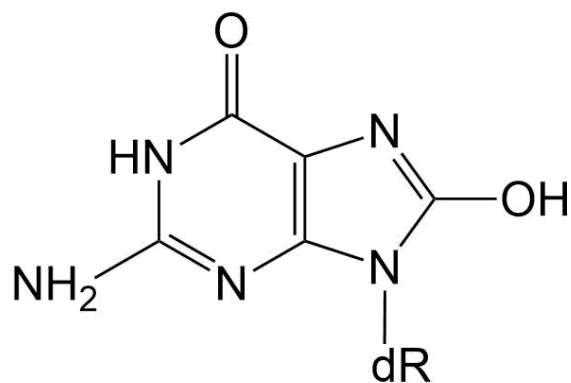
A classic example of xenobiotic bioactivation to a free radical that initiates lipid peroxidation is the cytochrome P450-dependent conversion of carbon tetrachloride ( $\text{CCl}_4$ ) to generate the trichloromethyl radical ( $\cdot\text{CCl}_3$ ) and then the trichloromethyl peroxy radical  $\text{CCl}_3\text{OO}\cdot$ . Also the cytotoxicity of free iron is attributed to its function as an electron donor for the Fenton reaction (see section on [Oxidative stress I](#)) for instance via the generation of superoxide anion radicals by paraquat redox cycling) leading to the formation of the highly reactive hydroxyl radical, a known initiator of lipid peroxidation.

#### Oxidative DNA damage

ROS can also oxidize DNA bases and sugars, produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications and DNA crosslinks. A common modification to DNA is the hydroxylation of DNA bases leading to the formation of oxidized DNA adducts. Although these adducts have been identified in all four DNA bases, guanine is the most susceptible to oxidative damage because it has the lowest oxidation potential of all of the DNA bases. The oxidation of guanine and by hydroxyl radicals leads to the formation 8-hydroxyguanosine (8-OH-dG) (Figure 4).



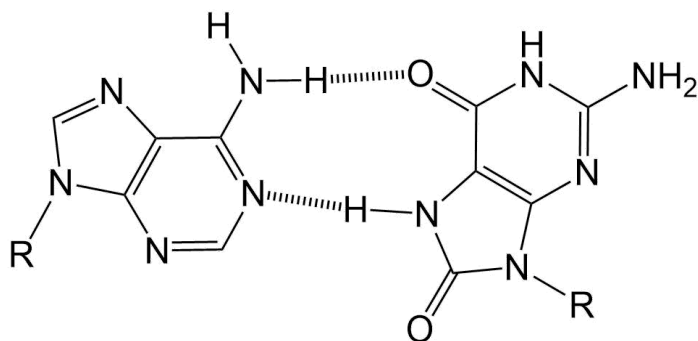
Guanosine



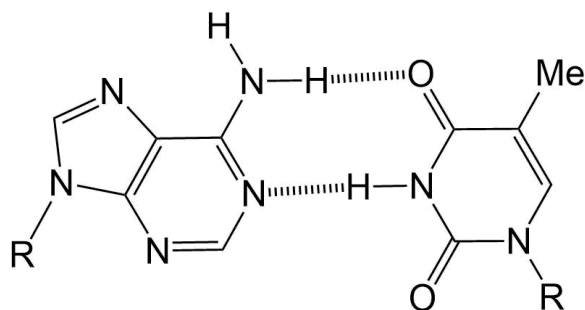
8-Hydroxyguanosine

Figure 4. The hydroxylation of guanine. Drawn by Steven Droge.

Oxidation of guanine has a detrimental effect on base pairing, because instead of hydrogen bonding with cytosine as guanine normally does, it can form a base pair with adenine. As a result, during DNA replication, DNA polymerase may mistakenly insert an adenosine opposite to an 8-oxo-2'-deoxyguanosine (8-oxo-dG), resulting in a stable change in DNA sequence, a process known as mutagenesis (Figure 5).



A-O8G base pair



A-T base pair

Figure 5. Base pairing with 8-oxo-2'-deoxyguanosine (8-oxo-dG). Drawn by Steven Droge.

Fortunately, there is an extensive repair mechanism that keeps mutations to a relatively low level. Nevertheless, persistent DNA damage can result in replication errors, transcription induction or inhibition, induction of signal transduction pathways and genomic

instability, events that are possibly involved in carcinogenesis (Figure 6). It has to be mentioned that mitochondrial DNA, is more susceptible to oxidative base damage compared to nuclear DNA due to its proximity to the electron transport chain (a source of ROS), and the fact that mitochondrial DNA is not protected by histones and has a limited DNA repair system.

Figure in preparation

**Figure 6.** Oxidative damage by ROS leading to mutations and eventually to tumour formation. Figure adapted from Boelsterli (2002).

One group of xenobiotics that have clearly been associated with eliciting oxidative DNA damage and cancer are redox-active metals, including Fe(III), Cu(II), Ag(I), Cr(III), Cr(VI), which may entail, as seen before, the production of hydroxyl radicals. Other (non-redox-active) metals that can induce ROS-formation themselves or participate in the reactions leading to endogenously generated ROS are Pb(II), Cd(II), Zn(II), and the metalloid As(III) and As(V). Compounds like polycyclic aromatic hydrocarbons (PAHs), likely the largest family of pollutants with genotoxic effects, require activation by endogenous metabolism to become reactive and capable of modifying DNA. This activation is brought about by the so-called Phase I biotransformation (see Section on [Xenobiotic metabolism and defence](#)).

Genetic detoxifying enzymes, like cytochrome P-450A1, are able to hydrophylate hydrophobic substrates. Whereas this reaction normally facilitates the excretion of the modified substance, some polycyclic aromatic hydrocarbons (PAHs), like benzo[a]pyrene generate semi stable epoxides that can ultimately react with DNA forming mutagenic adducts (see Section on [Xenobiotic metabolism and defence](#)). The main regulator of phase I metabolism in vertebrates, the Aryl hydrocarbon receptor (AhR), is a crucial player in this process. Some PAHs, dioxins, and some PCBs (the so-called coplanar congeners; see section on [Complex mixtures](#)) bind and activate AhR and increase the activity of phase I enzymes, including cytochrome P-450A1 (CYP1A1), by several fold. This increased oxidative metabolism enhances the toxic effects of the substances leading to increased DNA damage and inflammation (Figure 7).

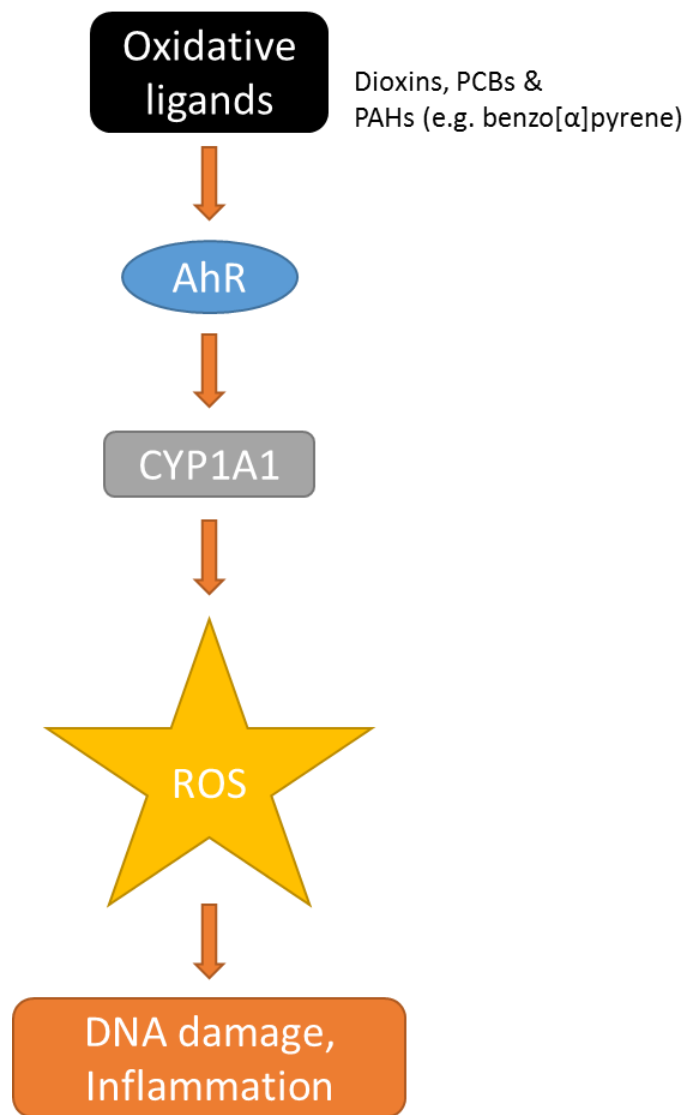


Figure 7. Environmental pollutants such as Dioxins, PCBs, PAHs (such as benzo[a]pyrene) bind to AhR and induce ROS production, DNA damage, and inflammatory cytokine production. Drawn by Frank van Belleghem.

### Oxidative effects on cell growth regulation

ROS production and oxidative stress can act both on cell proliferation and apoptosis. It has been demonstrated that low levels of ROS influence signal transduction pathways and alter gene expression.

Figure in preparation

**Figure 8.** Role of ROS in altered gene expression. Figure adapted from Klaassen (2013).

Many xenobiotics, by increasing cellular levels of oxidants, alter gene expression through activation of signaling pathways including cAMP-mediated cascades, calcium-calmodulin pathways, transcription factors such as AP-1 and NF-κB, as well as signaling through mitogen activated protein (MAP) kinases (Figure 8). Activation of these signaling cascades ultimately leads to altered gene expression or a number of genes including those affecting proliferation, differentiation, and apoptosis.

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#### 4.2.3.II. Question 1

The herbicide paraquat induces oxidative stress due to

- Interaction with the electron transport chain.

Its involvement in the redox cycle.

It interacts with glutathione.

Its involvement in the Fenton reaction.

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#### 4.2.3.II. Question 2

Which biopolymers can undergo damage from reactive oxygen species?

only DNA and proteins

only DNA and membranes

only proteins and membranes

DNA, proteins and membranes

---

#### 4.2.3.II. Question 3

Given: The three steps of lipid peroxidation:

I initiation

II propagation

III termination

Question: In which step(s) is  $O_2$  involved as a reagent or as a product?

Only I

Only II

I and II

II and III

---

#### 4.2.3.II. Question 4

Mitochondrial DNA, compared to nuclear DNA, is relatively susceptible to oxidative base damage.

Which of the given alternatives is *not* correct?

The increased susceptibility of mitochondrial DNA is due to:

The proximity of mitochondrial DNA to the electron transport chain

Mitochondrial DNA is not protected by histones

The limited levels of antioxidative compounds inside mitochondria

The limited mitochondrial DNA repair system

---



#### 4.2.4. Cytotoxicity: xenobiotic compounds causing cell death

**Authors:** Frank Van Belleghem, Karen Smeets

**Reviewers:** Timo Hamers, Bas J. Blaauboer

**Learning objectives:**

You should be able to:

- name the main factors that cause cell death,
- describe the process of necrosis and apoptosis,
- describe the morphological differences between apoptosis and necrosis,
- explain what form of cell death is caused by chemical substances.

**Keywords:** cell death, apoptosis, necrosis, caspase activation, mitochondrial permeability transition

#### Description

Cytotoxicity or cell toxicity is the result of chemical-induced macromolecular damage (see the section on [Protein inactivation](#)) or receptor-mediated disturbances (see the section on [Receptor interactions](#)). Initial events such as covalent binding to DNA or proteins; loss of calcium control or oxidative stress (see the sections on [Oxidative stress I](#) and [II](#)) can compromise key cellular functions or trigger cell death. Cell death is the ultimate endpoint of lethal cell injury; and can be caused by chemical compounds, mediator cells (*i.e.* natural killer cells) or physical/environmental conditions (*i.e.* radiation, pressure, etc.). The multistep process of cell death involves several regulated processes and checkpoints to be passed before the cell eventually reaches a point of no return, leading to either programmed cell death or apoptosis, or to a more accidental form of cell death, called necrosis. This section describes the cytotoxic process itself, *in vitro* cytotoxicity testing is dealt with in the section on [Human toxicity testing - II. In vitro tests](#).

#### Chemical toxicity leading to cell death

Cells can actively maintain the intracellular environment within a narrow range of physiological parameters despite changes in the conditions of the surrounding environment. This internal steady-state is termed cellular homeostasis. Exposure to toxic compounds can compromise homeostasis and lead to injury. Cell injury may be direct (primary) when a toxic substance interacts with one or more target molecules of the cell (*e.g.* damage to enzymes of the electron transport chain), or indirect (secondary) when a toxic substance disturbs the microenvironment of the cell (*e.g.* decreased supply of oxygen or nutrients). The injury is called reversible when cells can undergo repair or adaptation to achieve a new viable steady state. When the injury persists or becomes too severe, it becomes irreversible and the cell eventually perishes, thereby terminating cellular functions like respiration, metabolism, growth and proliferation, resulting in cell death (Niesink et al., 1996).

The main factors determining the occurrence of cell death are:

- the nature and concentration of the active toxic compound - in some cases a reactive intermediate - and the availability of that agent at the site of the target molecules;
- the role of the target molecules in the functioning of the cell and/or maintaining the microenvironment;
- the effectiveness of the cellular defence mechanisms in the detoxication and elimination of active agents, in repairing (primary) damage, and in the ability to induce proteins that either promote or inhibit the cell death process.

It is important to realize that also "harmless" substances such as glucose or salt may lead to cell injury and cell death by disrupting the osmotic homeostasis at sufficient concentrations. Even an essential molecule such as oxygen causes cell injury at sufficiently high partial pressures (see the sections on [Oxidative stress I](#) and [II](#)). Apart from that, all chemicals exert "baseline toxicity" (also called "narcosis") as described in the textbox "narcosis and membrane damage" in the section on [Toxicodynamics & Molecular Interactions](#).

The main types of cell death: necrosis and apoptosis

The two most important types of cell death are necrosis or accidental cell death (ACD) and apoptosis, a form of programmed cell death (PCD) or cell suicide. Cellular imbalances that initiate or promote cell death alone or in combination are oxidative stress, mitochondrial injury or disturbed calcium fluxes. These alterations are reversible at first, but after progressive injury, result in irreversible cell death. Cell death can also be initiated via receptor-mediated signal transduction processes. Apoptotic and necrotic cells differ in both the morphological appearance as well as biochemical characteristics. Necrosis is associated with cell swelling

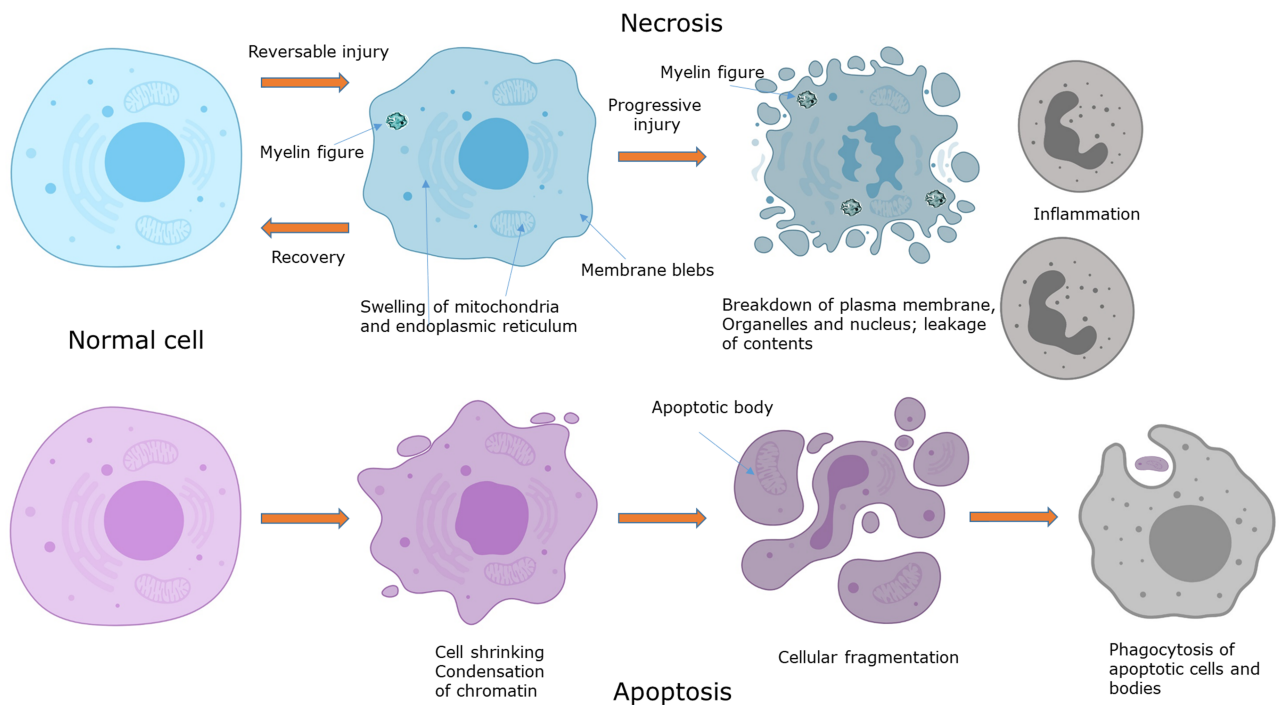
and a rapid loss of membrane integrity. Apoptotic cells shrink into small apoptotic bodies. Leaking cells during necrosis induce inflammatory responses, although inflammation is not entirely excluded during the apoptotic process (Rock & Kono, 2008).

### *Necrosis*

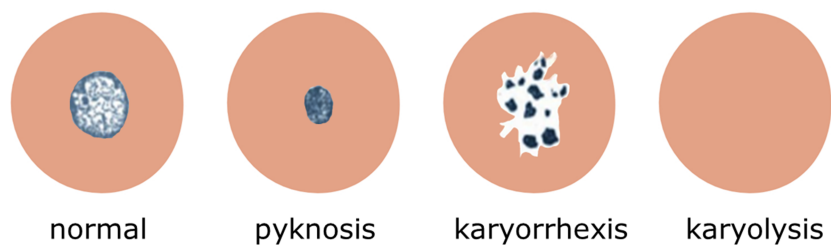
Necrosis has been termed accidental cell death because it is a pathological response to cellular injury after exposure to severe physical, chemical, or mechanical stressors. Necrosis is an energy-independent process that corresponds with damage to cell membranes and subsequent loss of ion homeostasis (in particular  $\text{Ca}^{2+}$ ). Essentially, the loss of cell membrane integrity allows enzymes to leak out of the lysosomal membranes, destroying the cell from the inside. Necrosis is characterized by swelling of cytoplasm and organelles, rupture of the plasma membrane and chromatin condensation (see Figure 1). These morphological appearances are associated with ATP depletion, defects in protein synthesis, cytoskeletal damage and DNA-damage. Besides, cell organelles and cellular debris leak via the damaged membranes into the extracellular space, leading to activation of the immune system and inflammation (Kumar et al., 2015). In contrast to apoptosis, the fragmentation of DNA is a late event. In a subsequent stage, injury is propagated across the neighbouring tissues via the release of proteolytic and lipolytic enzymes resulting in larger areas of necrotic tissue. Although necrosis is traditionally considered as an uncontrolled form of cell death, emerging evidence points out that the process can also occur in a regulated and genetically controlled manner, termed regulated necrosis (Berghe et al., 2014). Moreover, it can also be an autolytic process of cell disintegration after the apoptotic program is completed in the absence of scavengers (phagocytes), termed post-apoptotic or secondary necrosis (Silva, 2010).

### *Apoptosis*

Apoptosis is a regulated (programmed) physiological process whereby superfluous or potentially harmful cells (for example infected or pre-cancerous cells) are removed in a tightly controlled manner. It is an important process in embryonic development, the immune system and in fact, all living tissues. Apoptotic cells shrink and break into small fragments that are phagocytosed by adjacent cells or macrophages without producing an inflammatory response (Figure 3). It can be seen as a form of cellular suicide because cell death is the result of induction of active processes within the cell itself. Apoptosis is an energy-dependent process (it requires ATP) that involves the activation of caspases (cysteine-aspartyl proteases), pro-apoptotic proteins present as zymogens (*i.e.* inactive enzyme precursors that are activated by hydrolysis). Once activated, they function as cysteine proteases and activate other caspases. Caspases can be distinguished into two groups, the initiator caspases, which start the process, and the effector caspases, which specifically lyse molecules that are essential for cell survival (Blanco & Blanco 2017). Apoptosis can be triggered by stimuli coming from within the cell (intrinsic pathway) or from the extracellular medium (extrinsic pathway) as shown in Figure 2. The extrinsic pathway activates apoptosis in response to external stimuli, namely by extracellular ligands binding to cell-surface death receptors (Tumour Necrosis Factor Receptor ((TNFR)), leading to the formation of the death-inducing signalling complex (DISC) and the caspase cascade leading to apoptosis. The intrinsic pathway is activated by cell stressors such as DNA damage, lack of growth factors, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) burden, replication stress, microtubular alterations and mitotic defects (Galluzzi et al., 2018). These cellular events cause the release of cytochrome c and other pro-apoptotic proteins from the mitochondria into the cytosol via the mitochondrial permeability transition (MPT) pore. This is a megachannel in the inner membrane of the mitochondria composed of several protein complexes that facilitate the release of death proteins such as cytochrome c. The opening is triggered and tightly regulated by anti-apoptotic proteins, such as B-cell lymphoma-2 (Bcl-2) and pro-apoptotic proteins, such as Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist killer). The intrinsic and extrinsic pathways are regulated by the apoptosis inhibitor protein (AIP) which directly interacts with caspases and suppresses apoptosis. The release of the death protein cytochrome c induces the formation of a large protein structure formed in the process of apoptosis (the apoptosome complex) activating the caspase cascade leading to apoptosis. Other pro-apoptotic proteins oppose to Bcl (SMAC/Diablo) and stimulate caspase activity by interfering with AIP (HtrA2/Omi). HtrA2/Omi also activates caspases and endonuclease G (responsible for DNA degradation, chromatin condensation, and DNA fragmentation). The apoptosis-inducing factor (AIF) is involved in chromatin condensation and DNA fragmentation. Many xenobiotics interfere with the MPT pore and the fate of a cell depends on the balance between pro- and anti-apoptotic agents (Blanco & Blanco, 2017).



#### In-set 1: changes in the nucleus (depicted blue) during necrosis



#### In-set 2: changes in the nucleus during apoptosis

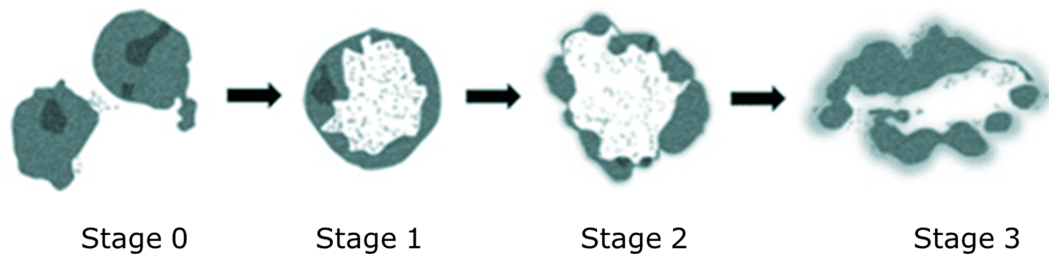


Figure 1. This diagram shows the observable differences between necrotic and apoptotic cell death. Reversible injury is characterized by cytoplasmic enlargement (oncosis), membrane blebbing, swelling of endoplasmic reticula and mitochondria, and the presence of myelin figures (twirled phospholipid masses from damaged cell membranes). Progressive injury leads to the necrotic breakdown of membranes, organelles and the nucleus. The nucleus can thereby undergo shrinking (pyknosis), fragmentation (karyorrhexis) or complete dissolution with loss of chromatin (karyolysis) (see in-set 1). The cell is eventually disrupted, releasing its contents and inducing an inflammatory reaction. In contrast, a cell undergoing apoptosis displays cell shrinkage, membrane blebbing, and (ring-shaped) chromatin condensation (see in-set 2, image adapted from Toné et al., 2007). The

nucleus and cytoplasm break up into fragments called apoptotic bodies, which are phagocytosed by surrounding cells or macrophages.

## Extrinsic pathway

## Intrinsic pathway

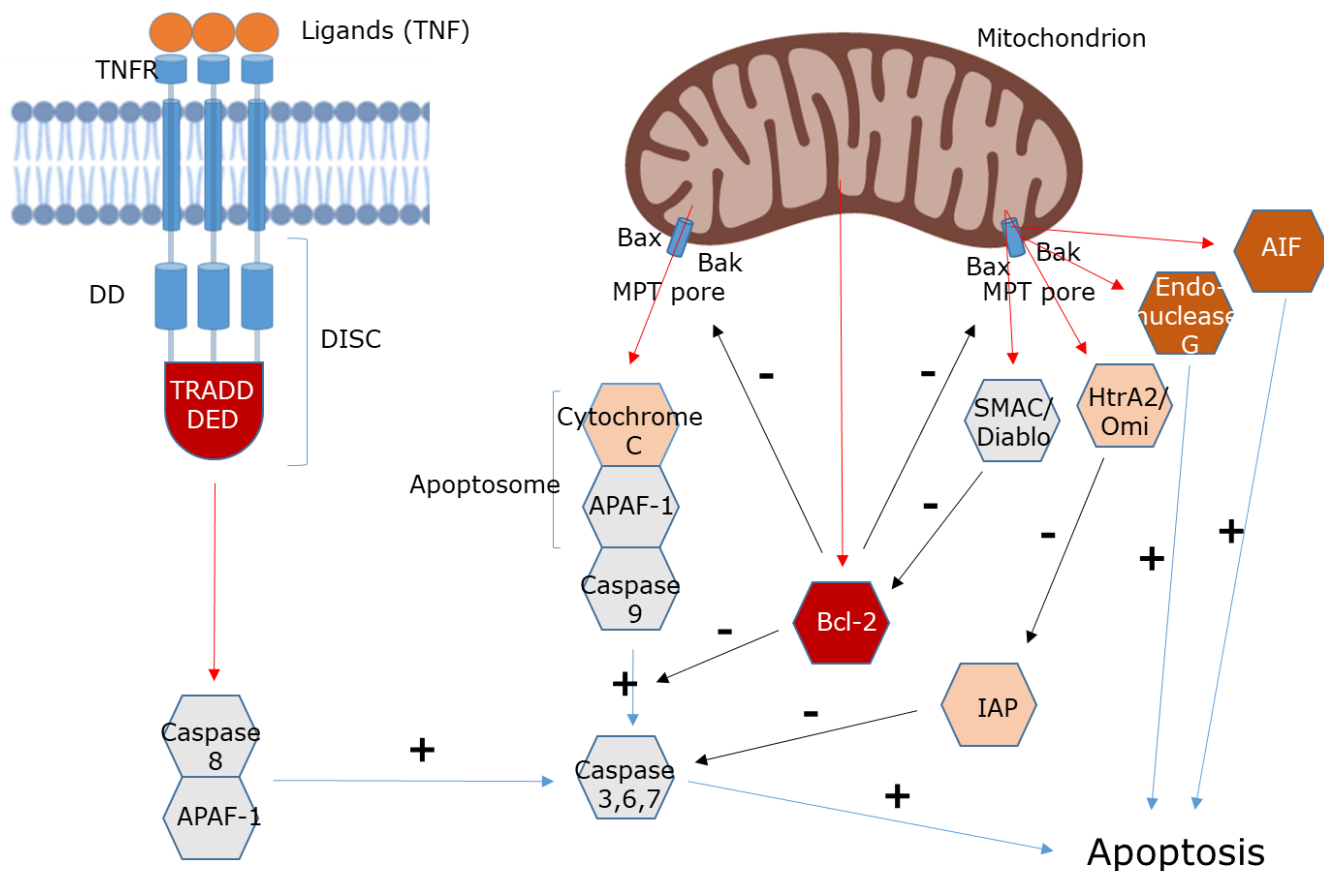


Figure 2. Scheme of the factors involved in the apoptotic process. APAF-1, Apoptosis protease activator factor-1; Bak, Bcl-associated antagonist killer; Bax, Bcl-associated X protein; Bcl-2, B cell lymphoma-2; DD, death domain; DED, death effector domain; IAP, inhibitor apoptosis protein; AIF, apoptosis-inducing factor; TNFR, tumour necrosis factor receptor; TRADD, TNFR-associated death domain. Image adapted from Blanco & Blanco 2017.

What determines the form of cell death caused by chemical substances?

Traditionally, toxic cell death was considered to be uniquely of the necrotic type. The classic example of necrosis is the liver toxicity of carbon tetrachloride ( $\text{CCl}_4$ ) caused by the biotransformation of  $\text{CCl}_4$  to the highly reactive radicals ( $\text{CCl}_3\cdot$  and  $\text{CCl}_3\text{OO}\cdot$ ).

Several environmental contaminants including heavy metals (Cd, Cu,  $\text{CH}_3\text{Hg}$ , Pb), organotin compounds and dithiocarbamates can exert their toxicity via induction of apoptosis, likely mediated by disruption of the intracellular  $\text{Ca}^{2+}$  homeostasis, or induction of mild oxidative stress (Orrenius et al., 2011).

In addition, some cytotoxic substances (e.g. arsenic trioxide ( $\text{As}_2\text{O}_3$ )) tend to induce apoptosis at low exposure levels or early after exposure at high levels, whereas they cause necrosis later at high exposure levels. This implicates that the severity of the insult determines the mode of cell death (Klaassen, 2013). In these cases, both apoptosis and necrosis involve the dysfunction of mitochondria, with a central role for the mitochondrial permeability transition (MPT). Normally, the mitochondrial membrane is impermeable to all solutes except for the ones having specific transporters. MPT allows the entry into the mitochondria of solutes with a molecular weight of lower than 1500 Daltons, which is caused by the opening of mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane. As these small-molecular-mass solutes equilibrate across the internal mitochondria membrane, the mitochondrial membrane potential ( $\Delta\Psi_{\text{mt}}$ ) vanishes (mitochondrial depolarization), leading to uncoupling of oxidative phosphorylation and subsequent adenosine triphosphate (ATP) depletion. Moreover, since proteins remain within the matrix at high concentration, the increasing colloidal osmotic pressure will result in movement of water into the matrix, which causes swelling of the mitochondria and rupture of the outer membrane. This results in the loss of intermembrane components (like

cytochrome c, AIF, HtrA2/Omi, SMAC/Diablo & Endonuclease G) to the cytoplasm. When MPT occurs in a few mitochondria, the affected mitochondria are phagocytosed and the cell survives. When more mitochondria are affected, the release of pro-apoptotic compounds will lead to the caspase activation resulting in apoptosis. When all mitochondria are affected, ATP becomes depleted and the cell will eventually undergo necrosis as shown in Figure 3 (Klaassen et al., 2013).

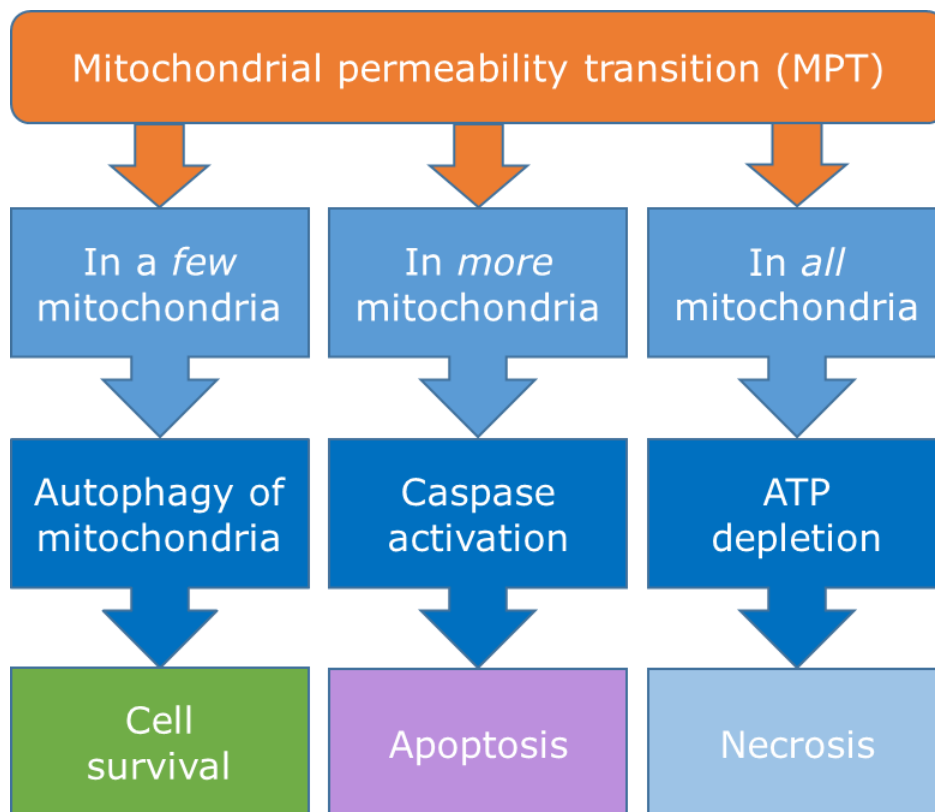


Figure 3. Dose-response relationship of toxicant-induced modes of cell death. The mode of cell death triggered by some toxicants is dose-dependent. Most often, exposure to low doses results in apoptosis, whereas higher levels of the same toxicant might cause necrosis. Image adapted from Klaassen et al., 2013.

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#### 4.2.4. Question 1

Which of the following is a characteristic of necrosis?

- The morphological changes are caused by release of lysosome enzymes.
- It is an energy-dependent process
- It is a programmed response to cellular injury.

It does not lead to inflammation

---

#### 4.2.4. Question 2

Which of the following is a characteristic of apoptosis?

Membrane bleb formation

Rapid loss of membrane integrity

Swelling of mitochondria

Cell shrinking

---

#### 4.2.4. Question 3

Which cellular organelles are involved in the initiation of the intrinsic pathway of apoptosis?

Ribosomes

Lysosomes

Mitochondria

Peroxisomes

---

#### 4.2.4. Question 4

Consider following statements:

- Secondary necrosis is a form of accidental cell death.
- MPT allows the entry of solutes leading to the increase of the volume of the cytoplasm.

Which statement is correct?

- Only I
- Only II
- Neither I, nor II
- Both I and II

---

#### 4.2.5. Neurotoxicity

**Author:** Jessica Legradi

**Reviewers:** Timo Hamers, Ellen Fritsche

### Learning objectives

You should be able to

- describe the structure of the nervous system
- explain how neurotransmission works
- mention some modes of action (MoA) by which pesticides and drugs cause neurotoxicity
- understands the relevance of species sensitivity to pesticides
- describe what developmental neurotoxicity (DNT) is

**Keywords:** Nervous system, Signal transmission, Pesticides, Drugs, Developmental Neurotoxicity

## Neurotoxicity

Neurotoxicity is defined as the capability of agents to cause adverse effects on the nervous system. Environmental neurotoxicity describes neurotoxicity caused by exposure to chemicals from the environment and mostly refers to human exposure and human neurotoxicity. Ecological neurotoxicity (eco-neurotoxicity) is defined as neurotoxicity resulting from exposure to environmental chemicals in species other than humans (e.g. fish, birds, invertebrates).

## The nervous system

The nervous system consists of the central nervous system (CNS) including the brain and the spinal cord and the peripheral nervous system (PNS). The PNS is divided into the somatic system (voluntary movements), the autonomic (sympathic and parasympathic) system and the enteric (gastrointestinal) system. The CNS and PNS are built from two types of nerve cells, i.e. neurons and glial cells. Neurons are cells that receive, process, and transmit information through electrical and chemical signals. Neurons consist of the soma with the surrounding dendrites and one axon with an axon terminal where the signal is transmitted to another cell (Figure 1A). Compared to neurons, glial cells can have very different appearances (Figure 1B), but are always found in the surrounding tissue of neurons where they provide metabolites, support and protection to neurons without being directly involved in signal transmission.

### Figure in preparation

**Figure 1.** Structures of a neuron (left; source: [https://simple.Wikipedia.org/wiki/Neuron](https://simple.wikipedia.org/wiki/Neuron)) and of glial cells (right)

Neurons are connected to each other via synapses. The sending neuron is called the presynaptic neuron whereas the receiving neuron is the postsynaptic neuron. In the synapse, a small space exists between the axon terminal of the presynaptic neuron and a dendrite of the postsynaptic neuron. This space is named synaptic cleft. Both neurons have ion channels that can be opened and closed in the area of the synapse. There are channels selective for chloride, sodium, calcium, potassium, or protons and non-selective channels. The channels can be voltage gated (i.e. they open and close depending on the membrane potential), ligand gated (i.e. they open and close depending on the presence of other molecules binding to the ion channel), or they can be stress activated (i.e. they open and close due to physical stress (stretching)). Ligands that can open or close ion channels are called neurotransmitters. Depending on the ion channel and if it opens or closes upon neurotransmitter binding, a neurotransmitter can inhibit or stimulate membrane depolarization (i.e. inhibitory or excitatory neurotransmitter, respectively). The ligands bind to the ion channel via receptors (link to section on [Receptor interaction](#)). Neurotransmitters have very distinct functions and are linked to physical processes like muscle contraction and body heat and to emotional/cognitive processes like anxiety, pleasure, relaxing and learning. The signal transmission via the synapse (i.e. neurotransmission) is illustrated in Figure 2.



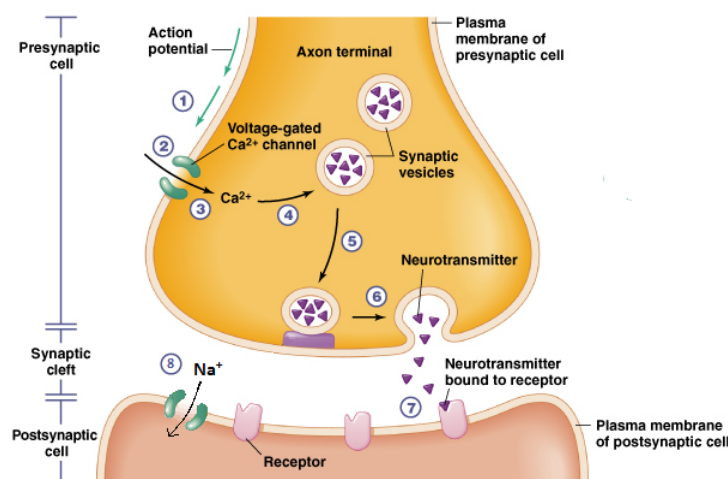


Figure 2: Synaptic neurotransmission by the excitatory neurotransmitter acetylcholine (ACh): 1. action potential arrives at presynaptic neuron; 2. stimulates opening of voltage-gated channels for  $\text{Ca}^{2+}$ ; 3.  $\text{Ca}^{2+}$  diffuses into the cytoplasm of the presynaptic cell; 4+5.  $\text{Ca}^{2+}$  causes vesicles containing ACh to move towards the presynaptic membrane; 6. ACh loaded vesicles **fuse** with the membrane, ACh is released and **diffuses across** the synaptic cleft; 7. ACh **temporarily binds** to receptor proteins on the postsynaptic membrane; causing ligand-gated ion channels for  $\text{Na}^+$  to open; 8.  $\text{Na}^+$  diffuses through postsynaptic membrane, **depolarizes the membrane** and generates an action potential. Source: <http://biology4alevel.blogspot.com/2016/06/122-synapses.html>

The cell membrane of a neuron contains channels that allow ions to enter and exit the neuron. This flow of ions is used to send signals from one neuron to the other. The difference in concentration of negatively and positively charged ions on the inner and outer side of the neuronal membrane creates a voltage across the membrane called the membrane potential. When a neuron is at rest (i.e. not signalling), the inside charge of the neuron is negative relative to the outside. The cell membrane is then at its resting potential. When a neuron is signalling, however, changes in ion inflow and outflow of ions lead to a quick depolarization followed by a repolarization of the membrane potential called action potential. A video showing how the action potential is produced can be found [here](#).

Neurons can be damaged via substances that damage the cell body (neuronopathy), the axon (axonopathy), or the myelin sheet or glial cells (myelopathy). Aluminum, arsenic, methanol, methylmercury and lead can cause neuropathy. Acrylamide is known to specifically affect axons and cause axonopathy.

## Neurotransmitter system related Modes of Action of neurotoxicity

Some of the modes of action relevant for neurotoxicity are disturbances of electric signal transmission and inhibition of chemical signal transmission, mainly through interference with the neurotransmitters. Pesticides are mostly designed to interfere with neurotransmission.

### 1. Interfering with Ion channels (see section on [Receptor interaction](#))

Pesticides such as DDT bind to open sodium channels in neurons, which prevents closing of the channels and leads to over-excitation. Pyrethroids, such as permethrin, increase the time of opening of the sodium channels, leading to similar symptoms. Lindane, cyclodiene insecticides like aldrin, dieldrin and endrin ("drins") and phenyl-pyrazols such as fipronil block GABA-mediated chloride channels and prevent hyperpolarization. GABA (gamma-aminobutyric acid) is an inhibitory neurotransmitter which is linked to relaxation and calming. It stimulates opening of chloride channels causing the transmembrane potential to become more negative (i.e. hyperpolarization), thereby increasing the depolarisation threshold for a new action potential. Blockers of GABA-mediated chloride channels prevent the hyperpolarizing effect of GABA, thereby decreasing the inhibitory effect of GABA. Neonicotinoids (e.g., imidacloprid) mimic the action of the excitatory neurotransmitter ACh by activating the nicotinic acetylcholine receptors (nAChR) in the postsynaptic membrane. These compounds are specifically designed for displaying a high affinity to insect nAChR.

Many human drugs, like sedatives also bind to neuro-receptors. Benzodiazepine drugs activate GABA-receptors causing hyperpolarization (activating GABA). Tetrahydrocannabinol (THC), which is the active ingredient in cannabis, activates the cannabinoid receptors also causing hyperpolarization. Compounds activating the GABA or cannabinoid receptors induce a strong feeling of relaxation. Nicotine binds and activates the AChR, which can help to concentrate.



## 2. AChE inhibition

Another very common neurotoxic mode of action is the inhibition of acetylcholinesterase (AChE). Organophosphate insecticides like dichlorvos and carbamate insecticides like propoxur bind to AChE, and hence prevent the degradation of acetylcholine in the synaptic cleft, leading to overexcitation of the post-synaptic cell membrane (see also section on [Protein interaction](#)).

## 3. Blocking Neurotransmitter uptake

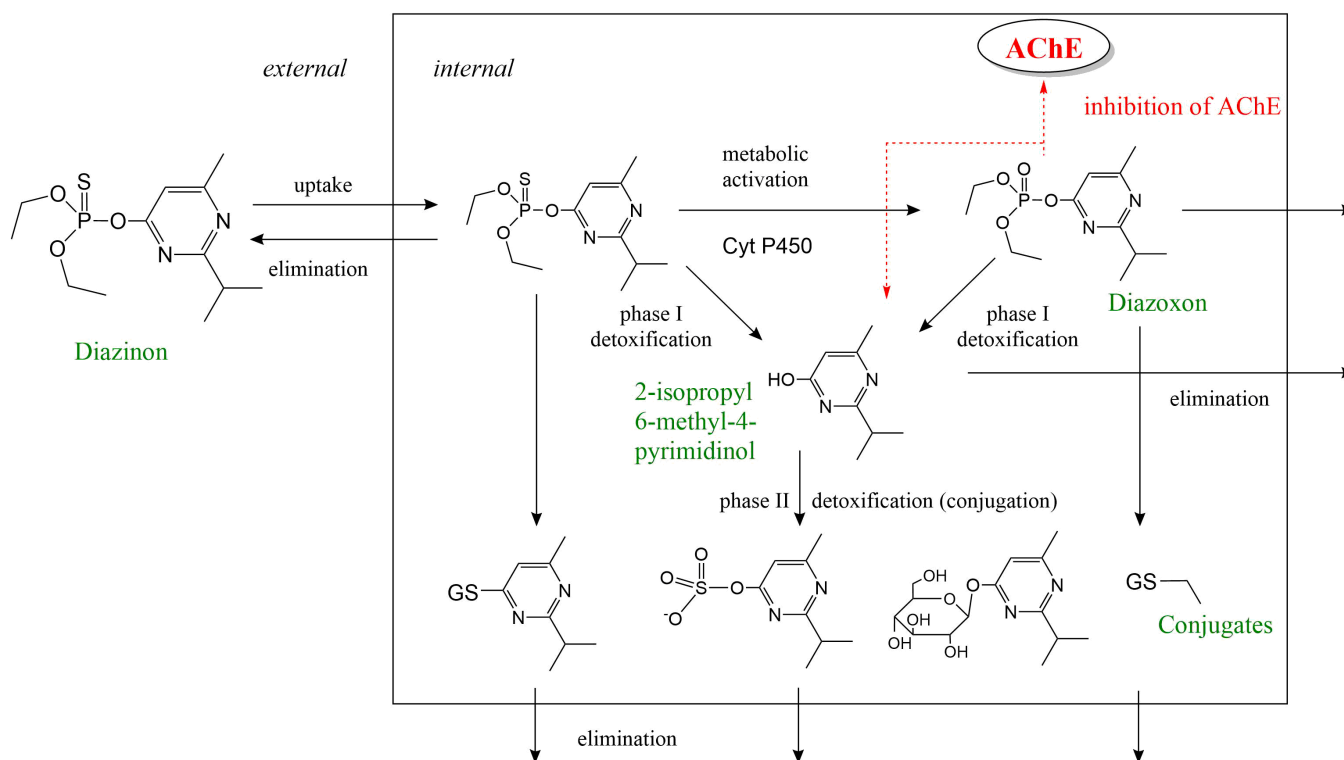
MDMA (3,4-methylenedioxyamphetamine, also known as ecstasy or XTC) and cocaine block the re-uptake of serotonin, norepinephrine and to a lesser amount dopamine into the pre-synaptic neuron, thereby increasing the amount of these neurotransmitters in the synaptic cleft. Amphetamines also increase the amount of dopamine in the cleft by stimulating the release of dopamine from the vesicles. Dopamine is a neurotransmitter which is involved in pleasure and reward feelings. Serotonin or 5-hydroxytryptamine is a monoamine neurotransmitter linked to feelings of happiness, learning, reward and memory.

## Long term exposure

When receptors are continuously activated or when neurotransmitter levels are continuously elevated, the nervous system adapts by becoming less sensitive to the stimulus. This explains why drug addicts have to increase the number of drugs taken to get to the desired state. If no stimulant is taken, withdrawal symptoms occur from the lack of stimulus. In most cases, the nervous system can recover from drug addiction.

## Species Sensitivity in Neurotoxicity

Differences in species sensitivity can be explained by differences in metabolic capacities between species. Most compounds need to be bio-activated, i.e. being biotransformed into a metabolite that causes the actual toxic effect. For example, most organophosphate insecticides are thio-phosphoesters that require oxidation prior to causing inhibition of AChE. As detoxification is the dominant pathway in mammals and oxidation is the dominant pathway in invertebrates, organophosphate insecticides are typically more toxic to invertebrates than to vertebrates (see Figure 3). Other factors important for species sensitivity are uptake and depuration rate.



**Figure 3:** Mechanism of action of an AChE inhibitor on the example of the insecticide diazinon. After oxidation catalyzed by cytochrome p450 monooxygenases, Diazinon is metabolized into diazoxon which can inhibit acetylcholinesterase. Via further phase I and phase II metabolism steps the molecule is eliminated. Drawn by Steven Droge.

## Developmental neurotoxicity

Developmental neurotoxicity (DNT) particularly refers to the effects of toxicants on the developing nervous system of organisms. The developing brain and nervous system are supposed to be more sensitive to toxic effects than the mature brain and nervous system. DNT studies must consider the temporal and regional occurrence of critical developmental processes of the nervous system, and the fact that early life exposure can lead to long-lasting neurotoxic effects or delays in neurological development. Species differences are also relevant for DNT. Here, developmental timing, speed, or cellular specificities might determine toxicity.

### 4.2.5. Question 1

What are the two major cell types found in the nervous system?

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### 4.2.5. Question 2

What does GABA do?

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### 4.2.5. Question 3

How does AChE inhibition work?

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### 4.2.5. Question 4

What makes invertebrates more sensitive to organophosphate insecticides?

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### 4.2.5. Question 5

Why is DNT important to study?

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## 4.2.6. Effects of herbicides

**Author:** Nico M. van Straalen

**Reviewers:** Cornelia Kienle, Henk Schat

### Learning objectives

You should be able to

- Explain the different ways in which herbicides are applied in modern agriculture
- Enumerate the eight major modes of action of herbicides
- Provide some examples of side-effects of herbicides

**Keywords:** Amino acid inhibitor, growth regulator, photosynthesis inhibitor, pre-emergence application, selectivity

## Introduction

Herbicides are pesticides (see section on [Crop protection products](#)) that aim to kill unwanted weeds in agricultural systems, and weeds growing on infrastructure such as pavement and train tracks. Herbicides are also applied to the crop itself, e.g. as a pre-harvest treatment in crops like potato and oilseed rape, to prevent growth of pathogens on older plants, or to ease mechanical harvest. In a similar fashion, herbicides are used to destroy grass of pastures in preparation of their conversion to cropland. These applications are designated "desiccation". Finally herbicides are used to kill broad-leaved weeds in pure grass-fields (e.g. golf courts).

Herbicides represent the largest volume of pesticides applied to date (about 60%), partly because mechanical and hand-executed weed control has declined considerably. The tendency to limit soil tillage (as a strategy to maintain a diverse and healthy soil life) has also stimulated the use of chemical herbicides.

Herbicides are obviously designed to kill plants and therefore act upon biochemical targets that are specific to plants. As the crop itself is also a plant, selectivity is a very important issue in herbicide application. This is achieved in several ways.

- Application of herbicides before emergence of the crop (pre-emergence application). This will keep the field free of weeds before germination, while the closed canopy of the crop prevents the later growth of weeds. This strategy is often applied in fast-growing crops that make a high canopy with a lot of shading on ground level, such as maize. Examples of herbicides used in pre-emergence application are glyphosate and metolachlor. Also the selectivity of seedling growth inhibitors such as EPTC is

due to the fact that these compounds are applied as part of a soil preparation and act on germinating plants before the crop emerges.

- Broad-leaved plants are more susceptible to herbicides that rely on contact with the leaves, because they intercept more of a herbicide spray than small-leaved plants such as grass. This type of selectivity allows some herbicides to be used in grassland and cereal crops, to control broad-leaved weeds; the herbicide itself is not intercepted by the crop. Examples are the chlorophenoxy-acetic acids such as MCPA and 2,4-D.
- In some cases the crop plant is naturally tolerant to a herbicide due to specific metabolic pathways. The selectivity of ACCase inhibitors such as diclofop-methyl, fenoxaprop-ethyl, and fluazifop-butyl is mostly due to this mechanism. These compounds inhibit acetyl-CoA carboxylases, a group of enzymes essential to fatty acid synthesis. However, in wheat the herbicidal compounds are quickly hydrolysed to non-toxic metabolites, while weeds are not capable of such detoxification. This allows such herbicides to be used in wheat fields. Another type of physiological selectivity is due to differential translocation, that is, some plants quickly transport the herbicide throughout the plant, enabling it to exert toxicity in the leaves, while others keep the substance in the roots and so remain less susceptible.
- Several crops have been genetically modified (gm) to become resistant to herbicides; one of the best-known modifications is the insertion of an altered version of the enzyme EPSP synthase. This enzyme is part of the shikimate pathway and is specifically inhibited by glyphosate (Figure 1). The modified version of the enzyme renders the plant insensitive to glyphosate, allowing herbicide use without damage to the crop. Various plant species have been modified in this way, although their culture is limited to countries that allow gm-crops (USA and many other countries, but not European countries).

### Classification by mode of action

The diversity of chemical compounds that have been synthesized to attack specific biochemical targets in plants is enormous. In an attempt to classify herbicides by mode of action a system of 22 different categories is often used (Sherwani et al. 2015). Here we present a simplified classification specifying only eight categories (Plant & Soil Sciences eLibrary 2019, Table 1).

**Table 1.** Classification of herbicides by mode of action

No.	Class (mode of action)	Examples of chemical groups	Example of active ingredient
1	Amino acid synthesis inhibitors	Sulfonylureas, imidazolones, triazolopyrimidines, epsp synthase inhibitors	Glyphosate
2	Seedling growth inhibitors	Carbamothiates, acetamides, dinitroanilines	EPTC
3	Growth regulators (interfere with plant hormones)	Phenoxy-acetic acids, benzoic acid, carboxylic acids, picolinic acids	2,4-D
4	Inhibitors of photosynthesis	Triazines, uracils, phenylureas, benzothiadiazoles, nitriles, pyridazines	Atrazine
5	Lipid synthesis inhibitors	Aryloxyphenoxypropionates, cyclohexanediones	Sethoxydim
6	Cell membrane disrupters	Diphenylethers, aryl triazolinones, phenylphthalamides, bipyridilium	Paraquat
7	Inhibitors of protective pigments	Isoxazolidones, isoxazoles, pyridazinones	Mesotrione
8	Unknown	Chemical compounds with proven herbicide efficacy but unknown mode of action	Ethofumesate

To illustrate the diversity of herbicidal mode of action, two examples of well-investigated mechanisms are highlighted here.

Plants synthesize aromatic amino acids using the shikimate pathway. Also bacteria and fungi avail of this pathway, but it is not present in animals. They must obtain aromatic amino acids through their diet. The first step in this pathway is the conversion of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to 5-enolpyruvylshikimate-3-phosphate (EPSP), by the enzyme EPSP synthase (Figure 1). EPSP is subsequently dephosphorylated and forms the substrate for the synthesis of aromatic amino acids such as phenylalanine, tyrosine and tryptophan.

Glyphosate bears a structural resemblance to PEP and competes with PEP as a substrate for EPSP synthase. However, in contrast to PEP it binds firmly to the active site of the enzyme and blocks its activity. The ensuing metabolic deficiency quickly leads to loss of growth potential of the plant.

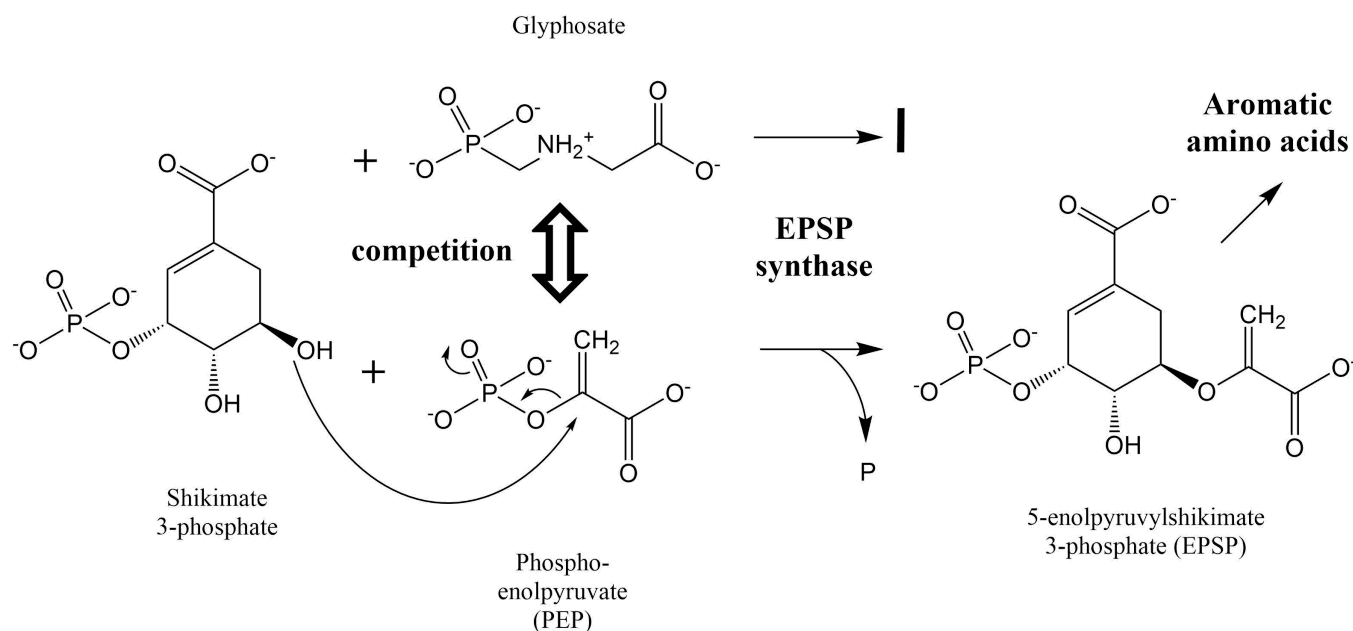


Figure 1. The first step in the shikimate pathway used by plants to synthesize aromatic amino acids. The enzyme EPSP synthase is inhibited by glyphosate due to competitive interaction with PEP. Redrawn by Steven Droge.

Another very well investigated mode of herbicidal action is photosynthesis inhibition by atrazine and other symmetrical triazines. In contrast to glyphosate, atrazine can only act in aboveground plants with active photosynthesis. Sunny weather stimulates the action of such herbicides. The action of atrazine is due to binding to the D1 quinone protein of the electron transport complex of photosystem II sitting in the inner membrane of the chloroplast (see [Figure 1 in Giardi and Pace, 2005](#)). Photosystem II (PSII) is a complex of macromolecules with light harvesting and antenna units, chlorophyll P680, and reaction centers that capture light energy and use it to split water, produce oxygen and transfer electrons to photosystem I, which uses them to eventually produce reduction equivalents. The D1 quinone has a "herbicide binding pocket" and binding of atrazine to this site blocks the function of PSII. A single amino acid in the binding pocket is critical for this; alterations in this amino acid provide a relatively easy possibility for the plant to become resistant to triazines.

## Side-effects

Most herbicides are polar compounds with good water solubility, which is a crucial property for them to be taken up by plants. This implies that herbicides, especially the more persistent ones, tend to leach to groundwater and surface water and are sometimes also found in drinking water resources. Given the large volumes applied in agriculture, concern has arisen that such compounds, despite them being designed to affect only plants, might harm other, so called "non-target" organisms.

In agricultural systems and their immediate surroundings, complete removal of weeds will reduce plant biodiversity, with secondary effects on plant-feeding insects and insectivorous birds. In the short term however herbicides will increase the amount of dead plant remains on the soil, which may benefit invertebrates that are less susceptible to the herbicidal effect, and find shelter in plant litter and feed on dead organic matter. Studies show that there is often a positive effect of herbicides on Collembola, mites and other surface-active arthropods (e.g. Fratello et al. 1985). Other secondary effects may occur when herbicides reach field-

bordering ditches, where suppression of macrophytes and algae can affect populations of macro-invertebrates such as gammarids and snails.

Direct toxicity to non-target organisms is expected from broad-spectrum herbicides that kill plants due to a general mechanism of toxicity. This holds for paraquat, a bipyridilium herbicide (cf. Table 1) that acts as a contact agent and rapidly damages plant leaves by redox-cycling; enhanced by sunshine, it generates oxygen radicals that disrupt biological membranes. Paraquat is obviously toxic to all life and represents an acute hazard to humans. Consequently, its use as a herbicide is forbidden in the EU since 2007.

In other cases the situation is more complex. Glyphosate, the herbicide with by far the largest application volume worldwide is suspect of ecological side-effects and has even been labelled "a probable carcinogen" by the IUCR (Tarazona et al., 2017). However, glyphosate is an active ingredient contained in various herbicide formulations, e.g. Roundup Ready, Roundup 360 plus, etc. Evidence indicates that most of the toxicity attributed to glyphosate is actually due to adjuvants in the formulation, specifically polyethoxylated tallowamines (Mesnage et al., 2013).

Another case of an unexpected side-effect from a herbicide is due to atrazine. In 2002 a group of American ecologists (Hayes et al., 2002) reported that the incidence of developmental abnormalities in wild frogs was correlated with the volume of atrazine sold in the area where frogs were monitored, across a large number of sites in the U.S. Male *Rana pipiens* exposed to atrazine in concentrations higher than 0.1 µg/L during their larval stages showed an increased rate of feminization, i.e. the development of oocytes in the testis. This would be due to induction of aromatase, a cytochrome P450 activity responsible for the conversion of testosterone to estradiol.

Finally the development of resistance may also be considered an undesirable side-effect. There are currently (2018) 499 unique cases (255 species of plant, combined with 167 active ingredients) of herbicide resistance, indicating the agronomical seriousness of this issue. A full discussion of this topic falls, however, beyond the scope of this module.

## Conclusions

Herbicides are currently an indispensable, high-volume component of modern agriculture. They represent a very large number of chemical groups and different modes of action, often plant-specific. While some of the older herbicides (paraquat, atrazine, glyphosate) have raised concern regarding their adverse effects on non-plant targets, the development of new chemicals and the discovery of new biochemical targets in plant-specific metabolic pathways remains an active field of research.

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### 4.2.6. Question 1

Define what is meant by a pre-emergence herbicide and why this is useful in agronomy?

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### 4.2.6. Question 2

With a herbicide application in agriculture you want to kill unwanted plants among a crop that is in itself also a plant. How is this possible?

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### 4.2.6. Question 3

Enumerate the eight major modes of action of herbicides

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#### 4.2.6. Question 4

Can herbicides cause adverse effects on non-plant species?

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#### 4.2.7. Chemical carcinogenesis and genotoxicity

**Author:** Timo Hamers

**Reviewer:** Frederik-Jan van Schooten

##### Learning objectives

You should be able to

- describe the three different phases in cancer development and understands how compounds can stimulate the corresponding processes in these phases
- explain the difference between base pair substitutions and frameshift mutations both at DNA and the protein level
- describe the principle of bioactivation, which distinguishes indirect from direct mutagenic substances
- explain the difference between mutagenic and non-mutagenic carcinogens

**Key words:** Bioactivation; Mutation; Tumour promotion; Tumour progression; Ames test

#### Chemical carcinogenesis

Cancer is a collective name for multiple diseases sharing a common phenomenon that cell division is out of the control by growth-regulating processes. The consequent, autonomic growing cells are usually concentrated in a neoplasm (often referred to a tumour) but can also be diffusely dispersed, for instance in case of leukaemia or a mesothelioma. Benign tumours refer to neoplasms that are encapsulated and do not distribute through the body, whereas malign tumours cause metastasis, i.e. spreading of carcinogenic cells through the body causing new neoplasms at distant. The term benign sounds more friendly than it actually is: benign tumours can be very damaging to organs which are limited in available space (e.g. the brain in the skull) or to organs that can be obstructed by the tumour (e.g. the gut system).

The process of developing cancer (carcinogenesis) is traditionally divided in three phases, i.e.

1. the **initiation** phase, in the genetic DNA of a cell is permanently changed, resulting in daughter cells that genetically differ from their parent cells;
2. the **promotion** phase, in which the cell loses its differentiation and gains new characteristics causing increased proliferation;
3. the **progression** phase, in which the tumour invades surrounding tissues and causes metastasis.

Chemical carcinogenesis means that a chemical substance is capable of stimulating one or more of these phases. Carcinogenic compounds are often named after the phase that they affect, i.e. initiators (also called mutagens), tumour promoters, and tumour progressors. It is important to realize that many substances and processes naturally occurring in the body can also stimulate the different phases, i.e. inflammation and exposure to sun light may cause mutations, some endogenous hormones can act as very active promoters in hormone-sensitive cancers, and spontaneous mutations may stimulate the tumour progression phase.

#### Point mutations

Gene mutations (aka point mutations) are permanent changes in the order of the nucleotide base-pairs in the DNA. Based on what happens at the DNA level, point mutations can be divided in three types, i.e. a replacement of an original base-pair by another base-pair (**base-pair substitution**), the **insertion** of an extra base-pair or the **deletion** of an original base-pair (Figure 1). In a coding part of DNA, three adjacent nucleotides on a DNA strand (i.e. a triplet) form a codon that encodes for an amino acid in the ultimate protein. Because insertions and deletions cause a shift in these triplet reading frames with one nucleotide to the left or to the right, respectively, these point mutations are also called **frame-shift mutations**.

Based on what happens at the protein level for which a gene encodes, point mutations can also be divided into three types. A **missense** mutation means that the mutated gene encodes for a different protein than the wildtype gene, a **nonsense** mutation means that the mutation introduces a STOP codon that interrupts gene transcription resulting in a truncated protein, and a **silent** mutation means that the mutated gene still encodes for exactly the same protein, despite the fact that the genetic code has been changed. Silent mutations are always base-pair substitutions, because the triplet structure of the DNA has not been damaged.

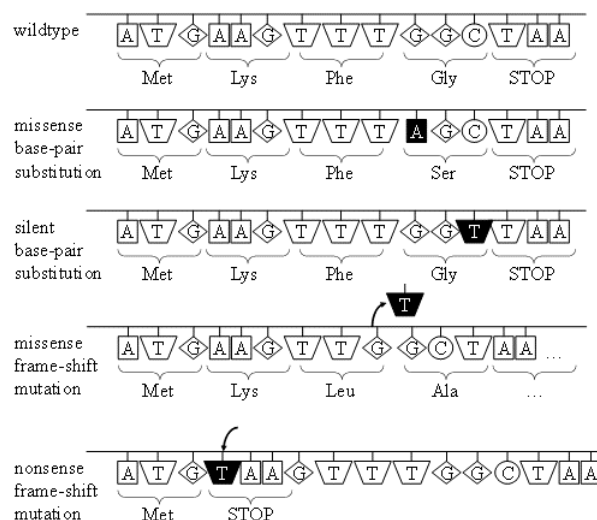


Figure 1: Examples of missense, nonsense en silent mutations at the polypeptide level, based on base-pair substitutions en frame-shift mutations at the genomic DNA level.

A very illustrative example of the difference between a base-pair substitution and a frameshift mutation at the level of protein expression is the following "wildtype" sentence, consisting of only three letter words representing the triplets in the genomic DNA:

***The fat cat ate the hot dog.***

Imagine that the letter t in cat is replaced by an r due to a base-pair substitution. The sentence then reads:

***The fat car ate the hot dog.***

This sentence clearly has another meaning, i.e. it contains missense information.

Imagine now that the letter a in fat is replaced by an e due to a base-pair substitution. The sentence then reads:

***The fet cat ate the hot dog.***

This sentence clearly contains a spelling error (i.e. a mutation), but it's meaning has not changed, i.e. it contains a silent mutation.

Imagine now that an additional letter m causes a frameshift in the word fat, due to an insertion. The sentence then reads:

***The fma tca tat eth eho tdo.***

This sentence clearly has another meaning, i.e. it contains missense information.

Similarly, leaving out the letter a in fat also causes a frameshift mutation, due to a deletion. The sentence then reads:

***The ftc ata tet heh otd og.***

Again, this sentence clearly has another meaning, i.e. it contains missense information.

This example suggests that the consequences are more dramatic for a frameshift mutation than for a base-pair substitution. Please keep in mind that the replacement of a cat by a car may also have huge consequences in daily life!

## Mutagenic compounds

Base-pair substitutions are often caused by electrophilic substances that want to take up an electron from especially the nucleophilic guanine base that wants to donate an electron to form an electron pair. The consequent guanine addition product (adduct) forms a base-pair with thymine causing a base-pair substitution from G-C to A-T. Alternatively, the guanine adduct may split from the phosphate-sugar backbone of the DNA, leaving an "empty" nucleotide spot in the triplet that can be taken by any nucleotide during DNA replication. Alternatively, base-pair substitutions may be caused by reactive oxygen species (ROS), which are radical compounds that also take up an electron from guanine and form guanine oxidation products (for instance hydroxyl adducts). It should be realized that a DNA adduct can only cause an error in the order of nucleotides (i.e. a mutation) if it is present during DNA replication. Before a cell goes into the DNA synthesis phase of the cell cycle, however, the DNA is thoroughly checked, and possible errors are repaired by DNA repair systems.

Exposure to **direct mutagenic** electrophilic agents rarely occurs because these substances are so reactive that they immediately bind to proteins and DNA in our food and environment. Therefore, DNA damage by such substances in most cases originates from **indirect mutagenic** compounds, which are activated into DNA-binding agents during Phase I of the biotransformation. This process of **bioactivation** is a side-effect of the biotransformation, which is actually aiming at rapid detoxification and elimination of toxic compounds.



Frame-shift mutations are often caused by **intercalating** agents. Unlike electrophilic agents and ROS, intercalating agents do not form covalent bonds with the DNA bases. Instead, due to their planar structure intercalating agents fit exactly between two adjacent nucleotides in the DNA helix. As a consequence, they hinder DNA replication, causing the insertion of an extra nucleotide or the deletion of an original nucleotide in the replicate DNA strain.

#### **Ames test for mutagenicity**

Mutagenicity of a compound can be tested in the Ames test, named after Bruce Ames who developed the assay in the early 1970s. The assay makes use of a *Salmonella* bacteria strain that contains a mutation in a gene encoding for an enzyme involved in the synthesis of the amino acid histidine. Consequently, the bacteria can no longer produce histidine (become "his-") and become auxotrophic, i.e. they depend on their culture medium for histidine. In the assay, the bacteria are exposed to the test compound in a medium that does not contain histidine. If the test compound is not mutagenic, the bacteria cannot grow and will die. If the test compound is mutagenic, it may cause a back-mutation (reversion) of the original mutation in a few bacteria, restoring the autotrophic capacity of the bacteria (i.e. their capacity to produce their own histidine). Growth of mutated bacteria on the histidine depleted medium can be followed by counting colonies (on an agar plate) or by measuring metabolic activity (in a fluctuation assay). Direct mutagenic compounds can be tested in the Ames test without extra treatment. Indirect mutagenic compounds, however, have to be bio-activated before they exert their mutagenic action. For this purpose, a liver homogenate is added to the culture medium containing all enzymes and cofactors required for Phase-I biotransformation of the test compound. This liver homogenate with induced cytochrome P450 (cyp) activity is usually obtained from rats exposed to mixed-type of inducers (i.e. cyp1a, cyp2b, cyp3a), such as the PCB-mixture Aroclor 1254.

### Compounds involved in tumour promotion and tumour progression

As stated above, non-mutagenic carcinogens are involved in stimulating the tumour promotion. Tumour promoting substances stimulate cell proliferation and inhibit cell differentiation and apoptosis. Unlike mutagenic compounds, tumour promoting compounds do not interfere directly with DNA and their effect is reversible. Many endogenous substances (e.g. hormones) may act as tumour promoting agents.

The first illustration that chemicals may induce cancer comes from the case of the chimney sweepers in London around 1775. The surgeon Percival Pott (1714-1788) noticed that many adolescent male patients who had developed scrotal cancer had worked during their childhood as a chimney sweeper. Pott made a direct link between exposure to soot during childhood and development of cancer at later age. Based on this discovery, taking a shower after work became mandatory for children working as chimney sweepers, and the observed scrotum cancer incidence decreased. As such, Percival Pott was the first person (i) to link cancer development to chemical substances, (ii) to link early exposure to later cancer development, and (iii) to obtain better occupational health by decreased exposure through better hygiene. In retrospective, we now know that the mutagens involved were polycyclic aromatic hydrocarbons (PAHs) that were bio-activated into highly reactive diol-epoxide metabolites. The delay in cancer development after the early childhood exposure can be attributed to the absence of a tumour promotor. Only after the chimney sweepers had gone through puberty they had sufficient testosterone levels, which stimulates scrotum tissue growth and in this case acted as an endogenous tumour promoting agent.

Tumour progression is the result of aberrant transcriptional activity from either genetic or epigenetic alterations. Genetic alterations can be caused by substances that damage the DNA (called genotoxic substances) and thereby introduce strand breaks and incorrect chromosomal division after mitosis. This results in the typical instable chromosomal characteristics of a malign tumour cell, i.e. a karyotype consisting of reduced and increased numbers of chromosomes (called **aneuploidy** and **polyploidy**, respectively) and damaged chromosomal structures (**abberations**). Chemical substances causing aneuploidy are called aneugens and substances causing chromosomal abberations are called clastogens. Genotoxic substances are also very often mutagenic compounds. Multiple mutations in so-called proto-oncogenes and tumour suppressor genes are necessary to transform a normal cell into a tumour cell. In a healthy cell, cell proliferation is under control by proto-oncogenes that stimulate cell proliferation and tumour suppressor genes that inhibit cell proliferation. In a cancer cell, the balance between proto-oncogenes and tumour suppressor genes is disturbed: proto-oncogenes act as oncogenes, meaning that they continuously stimulate cell proliferation, due to mutations and polyploidy, whereas tumour suppressor genes have become inactive due to mutations and aneuploidy.

Epigenetic alterations are changes in the DNA, but not in its order of nucleotides. Typical epigenetic changes include changes in DNA methylation, histone modifications, and microRNA expression. Compounds that change the epigenome may stimulate tumour progression for instance by stimulating expression of oncogenes and inhibiting expression of tumour suppressor genes. The role in tumour promotion and progression of substances that are capable to induce epigenetic changes is a field of ongoing study.

#### 4.2.7. Question 1



What are the three characteristics of the different cancer development stages?

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#### 4.2.7. Question 2

What is the difference between a direct and an indirect mutagenic substance?

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#### 4.2.7. Question 3

Explain the principle of the Ames test?

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#### 4.2.7. Question 4

What is the difference between a base-pair substitution and a frameshift mutation?

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### 4.2.8. Endocrine disruption

**Author:** Majorie van Duursen

**Reviewer:** Timo Hamers, Andreas Kortenkamp

#### Learning objectives

You should be able to

- explain how xenobiotics can interact with the endocrine system and hormonal actions;
- describe the thyroid system and molecular targets for thyroid hormone disruption;
- explain the concept "it's the timing of the dose that makes the poison".

**Keywords:** Endocrine system; Endocrine Disrupting Chemical (EDC); DES; Thyroid hormone disruption; Multi- and transgenerational effects

#### Short history

The endocrine system plays an essential role in the short- and long-term regulation of a variety of biochemical and physiological processes, such as behavior, reproduction, growth as well as nutritional aspects, gut, cardiovascular and kidney function and the response to stress. As a consequence, chemicals that cause changes in hormone secretion or in hormone receptor activity may target many different organs and functions and may result in disorders of the endocrine system and adverse health effects. The nature and the size of endocrine effects caused by chemicals depend on the type of chemical, the level and duration of exposure as well as on the timing of exposure.

The "DES drug disaster" is one of the most striking examples that endocrine-active chemicals can have severe adverse health effects in humans. There was a time when the synthetic estrogen diethylstilbestrol (DES) was considered a miracle drug (Figure 1). DES was prescribed from the 1940s-1970s to millions of women around the world to prevent miscarriages, abortion and premature labor. However, in the early 1970s it was found that daughters of mothers who took DES during their pregnancy have an increased risk of developing a specific vaginal and cervical cancer type. Other studies later demonstrated that women who had been exposed to DES in the womb (in utero) also had other health problems, like increased risk of breast cancer, increased incidence of genital malformations, infertility, miscarriages, and complicated pregnancies. Now, even two generations later, babies are born with reproductive tract malformations that are suspected to be caused by this drug their great grandmothers took during pregnancy. The effects of DES are attributed to the fact that it is a synthetic estrogen (i.e. a xenobiotic compound having similar properties as the natural estrogen 17 $\beta$ -estradiol), thereby disrupting normal endocrine regulation as well as epigenetic processes during development (link to section on [Developmental Toxicity](#)).

Around the same time of the DES drug disaster, Rachel Carson wrote a New York Times best-seller called Silent Spring. The book focused on endocrine disruptive properties of persistent environmental contaminants, such as the insecticide DDT (Dichloro Diphenyl Trichloroethane). She wrote that these environmental contaminants were badly degradable in the environment and cause reproductive failure and population decline in a variety of wild life. At the time the book was published, endocrine disruption was a controversial scientific theory that was met with much scepticism as empirical evidence was largely lacking. Still, the book of Rachel Carson has encouraged scientific, societal and political discussions about endocrine disruption. In 1996, another popular scientific book was published that presented more scientific evidence to warn against the effects of endocrine disruption: Our Stolen Future: Are We Threatening Our Fertility, Intelligence, and Survival? A Scientific Detective Story by Theo Colborn, Dianne Dumanoski and John Peterson Myers.



Figure 1: Advertisement from the 1950s for desPLEX, a synthetic drug containing diethylstilbestrol.

Currently, endocrine disruption is a widely accepted concept and many scientific studies have demonstrated a wide variety of adverse health effects that are attributed to exposure to endocrine active compounds in our environment. Human epidemiological studies have shown dramatic increases in incidences of hormone-related diseases, such as breast, ovarian, testicular and prostate cancer, endometrial diseases, infertility, decreased sperm quality, and metabolic diseases. Considering that hormones play a prominent role in the onset of these diseases, it is highly likely that exposure to endocrine disrupting compounds contributes to these increased disease incidences in humans. In wildlife, the effects of endocrine disruption include feminizing and demasculinizing effects leading to deviant sexual behaviour and reproductive failure in many species, such as fish, frogs, birds and panthers. A striking example of endocrine disruption can be found in the lake Apopka alligator population. Lake Apopka is the third largest lake in the state of Florida, located a few kilometres north west of Orlando. In July 1980, heavy rainfall caused the spill of huge amounts of DDT in the lake by a local pesticide manufacturer. After that, the alligator population in Lake Apopka started to show a dramatic decline. Upon closer examination, these alligators had higher estradiol and lower testosterone levels in their blood, causing poorly developed testes and extremely small penises in the male offspring and severely malformed ovaries in females.

### What's in a name: EDC definition

Since the early discussions on endocrine disruption, the World Health Organisation (WHO) has published several reports to present the state-of-the-art in scientific evidence on endocrine disruption, associated adverse health effects and the underlying mechanisms. In 2002, the WHO proposed a definition for an endocrine disrupting compound (EDC), which is still being used. According to the WHO, an EDC can be defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations." In 2012, WHO stated that "EDCs have the capacity to interfere with tissue and organ development and function, and therefore they may alter susceptibility to different types of diseases throughout life. This is a global threat that needs to be resolved." The European Environment Agency concluded in 2012 that "chemically induced endocrine disruption likely affects human and wildlife endocrine health the world over." A recent report (Demeneix & Slama, 2019) that was commissioned by the European Parliament concluded that the lack of EDC consideration in regulatory procedures is "clearly detrimental for the environment, human health, society, sustainability and most probably for our economy".

### The endocrine system

Higher animals, including humans, have developed an endocrine system that allows them to regulate their internal environment. The endocrine system is interconnected and communicates bidirectionally with the neuro- and immunesystems. The endocrine system consist of glands that secrete hormones, the hormones themselves and targets that respond to the hormone. Glands that

secrete hormones include the pituitary, thyroid, adrenals, gonads and pancreas. There are three major classes of hormones: amino-acid derived hormones (e.g. thyroid hormones T3 and T4), peptide hormones (e.g. pancreatic hormones) and steroid hormones (e.g. testosterone and estradiol). Hormones elicit a wide variety of biological responses, which almost always start with binding of a hormone to a receptor in its target tissue. This will trigger a chain of intracellular events and eventually a physiological response. Understanding the chemical characteristics of a hormone and its function, may help explain the mechanisms by which chemicals can interact with the endocrine system and subsequently cause adverse health effects.

## Mechanism of action

Inherent to the complex nature of the endocrine system, endocrine disruption comes in many shapes and forms. It can occur at the receptor level (link to section on [Receptor Interaction](#)), but endocrine disruptors can also disturb the synthesis, metabolism or transport of hormones (locally or throughout the body), or display a combination of multiple mechanisms. For example, DDT can decrease testosterone levels via increased testosterone conversion by the aromatase enzyme, but also acts like an anti-androgen by blocking the androgen receptor and as an estrogen by activating the estrogen receptor. PCBs, polychlorinated biphenyls, are well-characterized thyroid hormone disrupting chemicals. PCBs are industrial chemicals that were widely used in transformers until their ban in the 1970s, but, due to their persistency, PCBs can still ubiquitously be found in the environment, human and wildlife blood and tissue samples (link to section on [POPs](#)). PCBs are known to interfere with the thyroid system via inhibition of thyroid hormone synthesis and/or increasing thyroid hormone metabolism, inhibiting binding of thyroid hormones to serum binding proteins, or blocking the ability of thyroid hormones to thyroid hormone receptors. These thyroid disrupting effects can occur in different organs throughout the body (see Figure 1 in Gilbert et al., 2012).

## The dose concept

In the 18<sup>th</sup> Century, physician and alchemist Paracelsus phrased the toxicological paradigm: "Everything is a poison. Only the dose makes that something is not a poison" (link to section [Concentration-response relationships](#), and to [Introduction](#)). Generally, this is understood as "the effect of the poison increases with the dose". According to this paradigm, upon determining the exposure levels where the toxic response begins and ends, safety levels can be derived to protect humans, animals and their environment. However, interpretation and practical implementation of this concept is challenged by issues that have arisen in modern-day toxicology, especially with EDCs, such as non-monotonic dose-response curves and timing of exposure.

To establish a dose-response relationship, traditionally, toxicological experiments are conducted where adult animals are exposed to very high doses of a chemical. To determine a safe level, you determine the highest test dose at which no toxic effect is seen (the NOAEL or no observed adverse effect level) and add an additional "safety" or "uncertainty" factor of usually 100. This factor 100 accounts for differences between experimental animals and humans, and differences within the human population (see [chapter 6 on Risk assessment](#)). Exposures below the safety level are generally considered safe. However, over the past years, studies measuring the effects of hormonally active chemicals also began to show biological effects of endocrine active chemicals at extremely low concentrations, which were presumed to be safe and are in the range of human exposure levels. There are several physiological explanations to this phenomenon. It is important to realize that endogenous hormone responses do not act in a linear, mono-tonic fashion (i.e. the effect goes in one direction), as can be seen in Figure 2 for thyroid hormone levels and IQ. There are feedback loops to regulate the endocrine system in case of over- or understimulation of a receptor and there are clear tissue-differences in receptor expression and sensitivity to hormonal actions. Moreover, hormones are messengers, which are designed to transfer a message across the body. They do this at extremely low concentrations and small changes in hormone concentrations can cause large changes in receptor occupancy and receptor activity. At high concentrations, the change in receptor occupancy is only minimal. This means that the effects at high doses do not always predict the effects of EDCs at lower doses and vice versa.

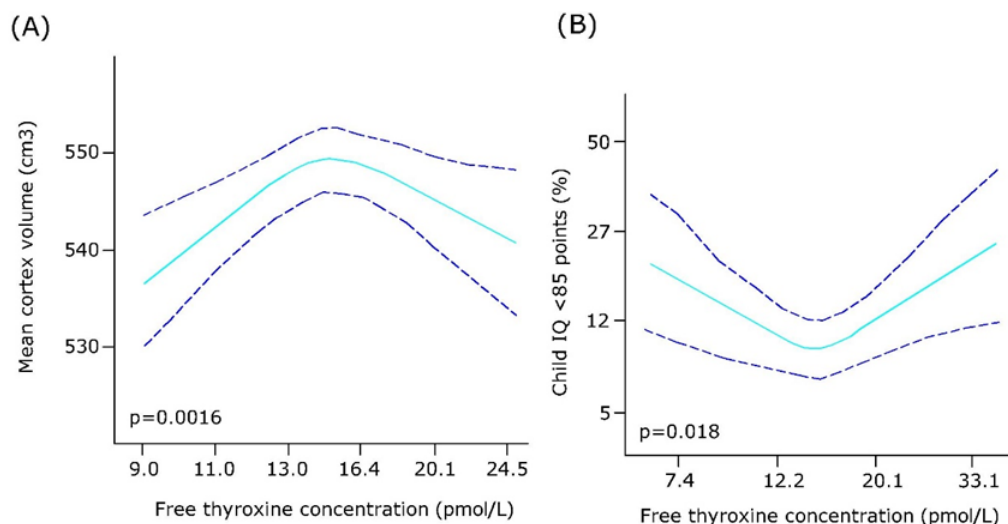


Figure 2. Relation between maternal thyroid hormone level (thyroxine) during pregnancy and (A) offspring cortex volume at the age of 8 years; (B) the predicted probability of offspring having an Intellectual Quotient (IQ) at the age of 6-8 years below 85 points. As women with overt hyperthyroidism or hypothyroidism were excluded, the range of values corresponds to those that can be considered within the normal limits for pregnancy of free thyroxine. Redrawn from Korevaar et al. (2016) by Wilma IJzerman.

It is becoming increasingly clear that not only the dose, but also the timing of exposure plays an important role in determining health effects of EDCs. Multi-generational studies show that EDC exposure in utero can affect future generations (Figure 3). Studies on the grandsons and granddaughters whose mothers were exposed prenatally to DES are limited as they are just beginning to reach the age when relevant health problems, such as fertility, can be studied. However, rodent studies with DES, bisphenol-A and DEHP show that perinatally exposed mothers have grandchildren with malformations of the reproductive tract as well as an increased susceptibility to mammary tumors in female offspring and testicular cancer and poor semen quality in male offspring. Some studies even show effects in the great-grandchildren (F3 generation), which indicates that endocrine disrupting effects have been passed to next generations without direct exposure of these generations. These are called trans-generational effects. Long-term, delayed effects of EDCs are thought to arise from epigenetic modifications in (germ) cells and can be irreversible and transgenerational (link to section on [Developmental Toxicity](#)). Consequently, safe levels of EDC exposure may vary, dependent on the *timing* of exposure. Adult exposure to EDCs is often considered activational, e.g. an estrogen-like compounds such as DES can stimulate proliferation of estrogen-sensitive breast cells in an adult leading to breast cancer. When exposure to EDCs occurs during development, the effects are considered to be organizational, e.g. DES changes germ cell development of perinatally exposed mothers and subsequently leads to genital tract malformations in their grandchildren. Multi-generational effects are clear in rodent studies, but are not so clear in humans. This is because it is difficult to characterize EDC exposure in previous generations (which may span over 100 years in humans), and it is challenging to filter out the effect of one specific EDC as humans are exposed to a myriad of chemicals throughout their lives.

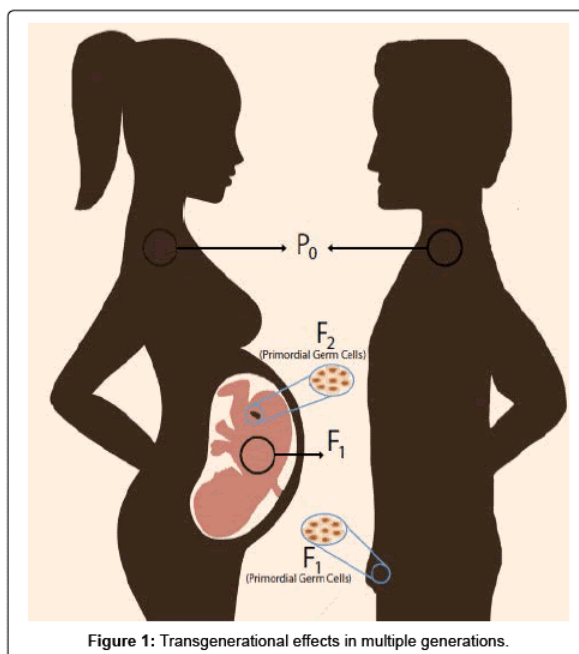


Figure 1: Transgenerational effects in multiple generations.

Figure 3: Exposure to EDCs can affect multiple generations. EDC exposure of parents ( $P_0$ ) can be multi-generational and lead to adverse health effects in children ( $F_1$ ) and grandchildren ( $F_2$ ). Some studies show adverse health effects in great-grandchildren ( $F_3$ ) upon exposure of the parent ( $P_0$ ). This is considered trans-generational, which means that no direct exposure of  $F_3$  has taken place, but that effects are passed on via epigenetic modifications in the germ cells of  $P_0$ ,  $F_1$  and/or  $F_2$ . Source: <https://www.omicsonline.org/open-access/epigenetic-effects-of-endocrine-disrupting-chemicals-2161-0525-1000381.php?aid=76673>

## EDCs in the environment

Some well-known examples of EDCs are pesticides (e.g. DDT), plastic softeners (e.g. phthalates, like DEHP), plastic precursors (e.g. bisphenol-A), industrial chemicals (e.g. PCBs), water- and stain-repellents (perfluorinated substances such as PFOS and PFOA) and synthetic hormones (e.g. DES). Exposure to EDCs can occur via air, housedust, leaching into food and feed, waste- and drinking water. Exposure is often unintentional and at low concentrations, except for hormonal drugs. Clearly, synthetic hormones can also have beneficial effects. Hormonal cancers like breast and prostate cancers can be treated with synthetic hormones. And think about the contraceptive pill that has changed the lives of many women around the world since the 1960s. Nowadays, no other method is so widely employed in so many countries around the world as the birth control pill, with an estimate of 75 million users among reproductive-age women with a partner. An unfortunate side effect of this is the increase in hormonal drug levels in our environment leading to feminization of male fish swimming in the polluted waters. Pharmaceutical hormones, along with naturally-produced hormones, are excreted by women and men and these are not fully removed through conventional wastewater treatments. In addition, several pharmaceuticals that are not considered to act via the endocrine system, can in fact display endocrine activity and cause reproductive failure in fish. These are for example the beta-blocker atenolol, antidiabetic drug metformin and analgesic paracetamol.

## Further reading:

WHO-IPCS. Global Assessment of the State-of-the-Science of Endocrine Disruptors. 2002  
[https://www.who.int/ipcs/publications/new\\_issues/endocrine\\_disruptors/en/](https://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/)

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European Environment Agency (EEA) The impacts of endocrine disruptors on wildlife, people and their environments The Weybridge+15 (1996-2011) report. EEA Technical report No 2/2012 EEA Copenhagen. ISSN 1725-2237  
<https://www.eea.europa.eu/publications/the-impacts-of-endocrine-disruptors>

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[http://www.europarl.europa.eu/RegData/etudes/STUD/2019/608866/IPOL\\_STU\(2019\)608866\\_EN.pdf](http://www.europarl.europa.eu/RegData/etudes/STUD/2019/608866/IPOL_STU(2019)608866_EN.pdf)

#### 4.2.8. Question 1

What are possible mechanisms to reduce the action of a certain hormone?

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#### 4.2.8. Question 2

Give three target sites for thyroid hormone disruption and name the biological process at that target site that can be affected by an EDC.

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#### 4.2.8. Question 3

What mechanism can cause demasculinization of male alligators by DDT?

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#### 4.2.8. Question 4

Why is timing of exposure important when assessing the risk of EDC exposure?

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### 4.2.9. Developmental toxicity

**Author:** Jessica Legradi, Marijke de Cock

**Reviewer:** Paul Fowler

#### Learning objectives

You should be able to

- explain the six principles of teratology
- name the difference between deformation, malformation and syndrome
- describe the principle of DoHAD
- indicate what epigenetics is and what epigenetic mechanisms could lead to transgenerational effects

**Keywords:** Teratogenicity, developmental toxicity, DoHAD, Epigenetics, transgenerational

### Developmental toxicity

Developmental toxicity refers to any adverse effects, caused by environmental factors, that interfere with homeostasis, normal growth, differentiation, or development before conception (either parents), during prenatal development, or postnatally until puberty. The effects can be reversible or irreversible. Environmental factors that can have an impact on development are lifestyle factors like alcohol, diet, smoking, drugs, environmental contaminants, or physical factors. Anything that can disturb the development of the embryo or foetus and produces a malformation is called a teratogen. Teratogens can terminate a pregnancy or produce adverse effects called congenital malformations (birth defects, anomaly). A malformation refers to any effect on the structural development of a foetus (e.g. delay, misdirection or arrest of developmental processes). Malformations occur mostly early in development and are permanent. Malformations should not be confused with deformations, which are mostly temporary effects caused by mechanical forces (e.g. moulding of the head after birth). One teratogen can induce several different malformations. All malformations caused by one teratogen are called a syndrome (e.g. fetal alcohol syndrome).

### Six Principles of Teratology (by James G. Wilson)

In 1959 James G. Wilson published the 6 principles of teratology. Till now these principles are still seen as the basics of developmental toxicology. The principles are:

#### 1. Susceptibility to teratogenesis depends on the genotype of the conceptus and a manner in which this interacts with adverse environmental factors

Species differences: different species can react different (sensitivities) to the same teratogen. For example, thalidomide (softenon) a drug used to treat morning sickness of pregnant woman causes severe limb malformations in humans whereas such effects were not seen in rats and mice.

Strain and intra litter differences: the genetic background of individuals within one species can cause differences in the response to a teratogen.



Interaction of genome and environment: organisms of the same genetic background can react differently to a teratogen in different environments.

Multifactorial causation: the summary of the above. The severity of a malformation depends on the interplay of several genes (inter and intra species) and several environmental factors.

## 2. Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence

During development there are periods where the foetus is specifically sensitive to a certain malformation (Figure 1). In general, the very early (embryonic) development is more susceptible to teratogenic effects.

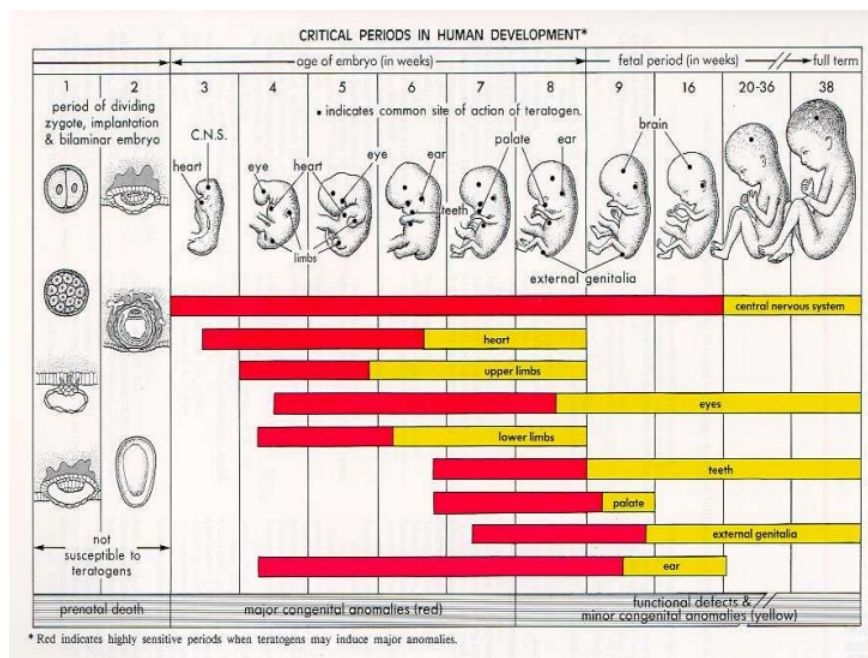


Figure 1: The critical (sensitive) periods during human development. During these periods tissues are more sensitive to malformations when exposed to a teratogen. The timing of period is different for different tissues. Source: <https://www.slideshare.net/SDRTL/critical-periods-in-human-development>

## 3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis)

Every teratogenic agent produces a distinctive malformation pattern. One example is the *foetal alcohol syndrome*, which induces abnormal appearance, short height, low body weight, small head size, poor coordination, low intelligence, behaviour problems, and problems with hearing or seeing and very characteristic facial features (increased distance between the eyes).

## 4. The access of adverse influences to developing tissues depends on the nature of the influence (agent)

Teratogens can be radiation, infections or chemicals including drugs. The teratogenic effect depends on the concentration of a teratogen that reaches the embryo. The concentration at the embryo is influenced by the maternal absorption, metabolism and elimination and the time the agent needs to get to the embryo. This can be very different between teratogens. For example, strong radiation is also a strong teratogen as it easily reaches all tissues of the embryo. This also means that a compound tested to be teratogenic in an *in vitro* test with embryos in a tube might not be teratogenic to an embryo in the uterus of a human or mouse as the teratogen may not reach the embryo at a critical concentration.

## 5. The four manifestations of deviant development are death, malformation, growth retardation and functional deficit

A teratogen can cause minor effects like functional deficits (e.g. reduced IQ), growth retardations or adverse effects like malformations or death. Depending on the timing of exposure and degree of genetic sensitivity, an embryo will have a greater or

lesser risk of death or malformations. Very early in development, during the first cell divisions, an embryo will be more likely to die rather than being implanted and developing further.

## 6. Manifestations of deviant development increase in frequency and degree as dosage increases, from the no-effect to the 100% lethal level

The number of effects and the severity of the effects increases with the concentration of a teratogen. This means that there is a threshold concentration below which no teratogenic effects occur (no effect concentration).

### Developmental Origins of Health and Disease (DOHaD)

The concept of Developmental Origins of Health and Disease (DOHaD) describes that environmental factors early in life contribute to health and disease later in life. The basis of this concept was the Barker hypothesis, which was formulated as an explanation for the rise in cardiovascular disease (CVD) related mortality in the United Kingdom between 1900 and 1950. Barker and colleagues observed that the prevalence of CVD and stroke was correlated with neo- and post-natal mortality (Figure 2). This led them to formulate the hypothesis that poor nutrition early in life leads to increased risk of cardiovascular disease and stroke later in life. Later, this was developed into the thrifty phenotype hypothesis stating that poor nutrition in utero programs for adaptive mechanisms that allow to deal with nutrient-poor conditions in later life, but may also result in greater susceptibility to metabolic syndrome. This thrifty hypothesis was finally developed into the DOHaD theory.

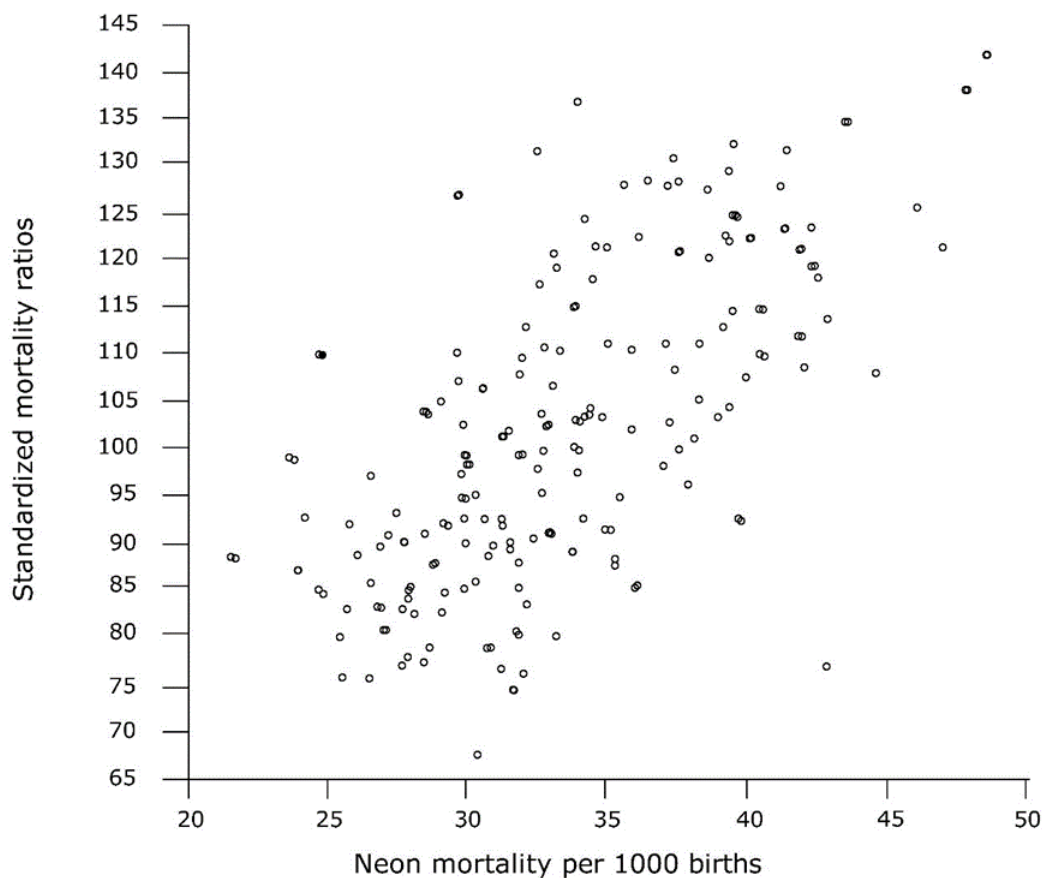


Figure 2: Standardized mortality ratios for ischaemic heart disease in both sexes (y-axis) and neonatal mortality per 1000 births, 1921-1925 (x-axis). Redrawn from Barker et al. (1986) by Wilma Ijzerman.

The effect of early life nutrition on adult health is clearly illustrated by the Dutch Famine Birth Cohort Study. In this cohort, women and men who were born during or just after the Dutch famine, were studied retrospectively. The Dutch famine was a famine that took place in the Western part of the German-occupied Netherlands in the winter of 1944-1945. Its 3-months duration creates the possibility to study the effect of poor nutrition during each individual trimester of pregnancy. Effects on birth weight, for example, may be expected if caloric intake during pregnancy is restricted. This was, however, only the case when the famine occurred during the second or the third trimesters. Higher glucose and insulin levels in adulthood were only seen for those exposed



in the third trimester, whereas those exposed during the second trimester showed a higher prevalence of obstructive airways disease. These effects were not observed for the other trimesters, which can be explained by the timing of caloric restriction during pregnancy: during normal pregnancy pancreatic islets develop during the third trimester, while during the second trimester the number of lung cells is known to double.

The DOHaD concept does not merely focus on early life nutrition, but includes all kinds of environmental stressors during the developmental period that may contribute to adult disease, including exposure to chemical compounds. Chemicals may elicit effects such as endocrine disruption or neurotoxicity, which can lead to permanent morphological and physiological changes when occurring early in life. Well-known examples of such chemicals are diethylstilbestrol (DES) and dichlorodiphenyltrichloroethane (DDT). DES was an estrogenic drug given to women between 1940 and 1970 to prevent miscarriage. It was withdrawn from the market in 1971 because of carcinogenic effects as well as an increased risk for infertility in children who were exposed in utero (link to section on [Endocrine disruption](#)). DDT is a pesticide that has been banned in most countries, but is still used in some for malaria control. Several studies, including a pooled analysis of seven European cohorts, found associations between in utero DDT exposure levels and infant growth and obesity.

The ubiquitous presence of chemicals in the environment makes it extremely relevant to study health effects in humans, but also makes it very challenging as virtually no perfect control group exists. This emphasizes the importance of prevention, which is the key message of the DOHaD concept. Adult lifestyle and corresponding exposure to toxic compounds remain important modifiable factors for both treatment and prevention of disease. However, as developmental plasticity, and therefore the potential for change, is highest early in life, it is important to focus on exposure in the early phases: during pregnancy, infancy, childhood and adolescence. This is reflected by regulators frequently imposing lower tolerable exposure levels for infants compared to adults.

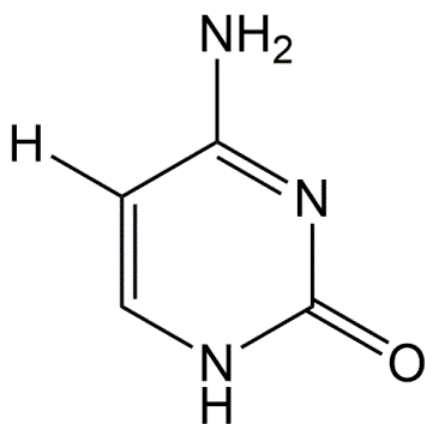
## Epigenetics

For some compounds in utero exposure is known to cause effects later in life (see DOHaD) or even induce effects in the offspring or grand-offspring of the exposed embryo (transgenerational effect). Diethylstilbestrol (DES) is a compound for which transgenerational effects are reported. DES was given to pregnant women to reduce the risk of spontaneous abortions and other pregnancy complications. Women who took DES during pregnancy have a slightly increased risk of breast cancer. Daughters exposed in utero, on the other hand, had a high tendency to develop rare vaginal tumours. In the third-generation, higher incidences of infertility, ovarian cancer and an increased risk of birth defects were observed. However, the data available for the third generation is small and therefore possess only limited evidence so far. Another compound suspected to cause transgenerational effects is the fungicide vinclozolin. Vinclozolin is an anti-androgenic endocrine disrupting chemical. It has been shown that exposure to vinclozolin leads to transgenerational effects on testis function in mice.

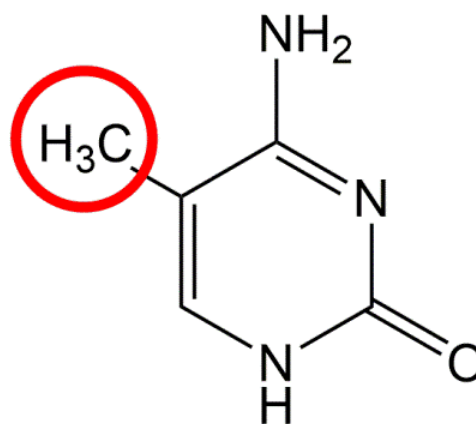
Transgenerational effects can be induced via genetic alterations (mutations) in the DNA. Thereby the order of nucleotides in the genome of the parental gametocyte is altered and this alteration is inherited to the offspring. Alternatively, transgenerational effects can be induced via epigenetic alterations. Epigenetics is defined as the study of changes in gene expression that occur without changes in the DNA sequence, and which are heritable in the progeny of cells or organisms. Epigenetic changes occur naturally but can also be influenced by lifestyle factors or diseases or environmental contaminants. Epigenetic alterations are a special form of developmental toxicology as effects might not cause immediate teratogenic malformations. Instead the effects may be visible only later in life or in subsequent generations. It is assumed that compounds can induce epigenetic changes and thereby cause transgenerational effects. For DES and vinclozolin epigenetic changes in mice have been reported and these might explain the transgenerational changes seen in humans. Two main epigenetic mechanisms are generally described as being responsible for transgenerational effects, i.e. DNA methylation and histone modifications.

## DNA methylation

DNA methylation is the most studied epigenetic modification and describes the methylation of cytosine nucleotides in the genome (Figure 3) by DNA methyltransferase (DNMTs). Gene activity generally depends on the degree of methylation of the promotor region: if the promotor is methylated the gene is usually repressed. One peculiarity of DNA methylation is that it can be wiped and replaced again during epigenetic reprogramming events to set up cell- and tissue-specific gene expression patterns. Epigenetic reprogramming occurs very early in development. During this phase epigenetic marks, like methylation marks are erased and remodelled. Epigenetic reprogramming is necessary as maternal and paternal genomes are differentially marked and must be reprogrammed to assure proper development.



Cytosine



methylated Cytosine

Figure 3: The methylation of the cytosine nucleotide. One hydrogen atom is replaced by a methyl group. Drawn by Steven Droge.

### Histone modification

Within the chromosome the DNA is densely packed around histone proteins. Gene transcription can only take place if the DNA packaging around the histones is loosened. Several histone modification processes are involved in loosening this packaging, such as acetylation, methylation, phosphorylation or ubiquitination of the histone molecules (Figure 4).

#### Figure in preparation

**Figure 4:** (a) The DNA is wrapped around the histone molecules. The histone molecules are arranged in a way that their amino acid tails are pointing out of the package. These tails can be altered for example via acetylation. (b) If the tails are acetylated the DNA is packed less tightly and genes can be transcribed. If the tails are not acetylated the DNA is packed very tight and gene transcription is hampered.

### References

Barker, D.J., Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1 (8489), 1077-1081.

#### 4.2.9. Question 1

Describe the difference between malformation and deformation?

#### 4.2.9. Question 2

What factors can influence the susceptibility of an organism to teratogen?

#### 4.2.9. Question 3

Describe how DOHaD differs from the Barker hypothesis?

#### 4.2.9. Question 4

What are the two epigenetic mechanisms mostly responsible for transgenerational effects?

#### 4.2.9. Question 5

Name a teratogenic compound?

#### 4.2.10. Immunotoxicity

**Author:** Nico van den Brink

**Review:** Manuel E. Ortiz-Santaliestra

#### Learning objectives:

You should be able to:

- Understand the complexity of potential effects of chemicals on the immune system
- Explain the difference between innate and acquired parts of the immune system
- Explain the most important modes of toxicity that may affect immune cells

**Key words:** Immune toxicology, pathogens, innate and adaptive immune system, lyme disease

#### Introduction

The immune system of organisms is very complex with different cells and other components interacting with each other. The immune system has the function to protect the organism from pathogens and infections. It consists of an innate part, which is active from infancy and an acquired part which is adaptive to exposure to pathogens. The immune system may include different components depending on the species (Figure 1).

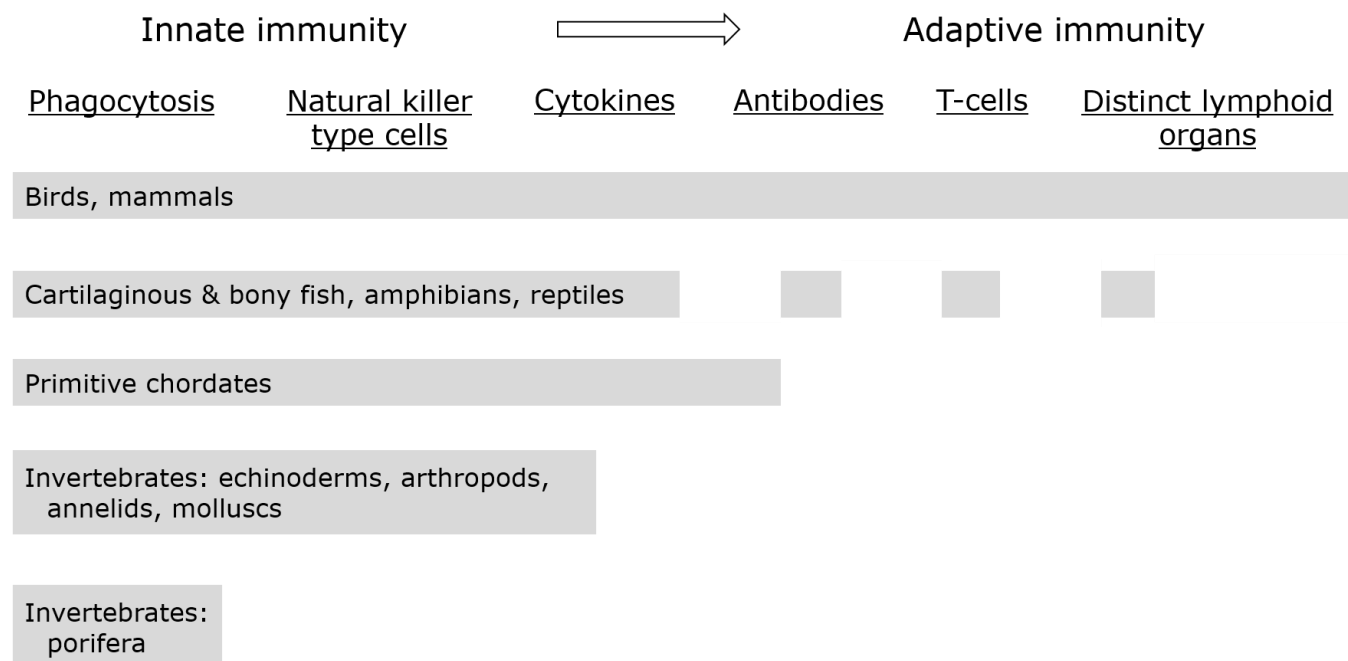


Figure 1. Simplified diagram of the evolution of the immune system indicating some preserved key immunological functions (adapted from Galloway and Handy, 2003).

The main organs involved in the immune system of mammals are spleen, thymus, bone marrow and lymph nodes. In birds, besides all of the above, there is also the bursa of Fabricius. These organs all play specific roles in the immune defence, e.g. the spleen synthesises antibodies and plays an important role in the dynamics of monocytes; the thymus is the organ where T-cells develop while in bone marrow lymphoid cells are produced, which are transported to other tissues for further development. The bursa of Fabricius is specific for birds and is essential for B-cell development. Blood is an important tissue to be considered because of its role in transporting cells. The innate system generally provides the first response to infections and pathogens, however it is not very specific. It consists of several cell types with different functions like macrophages, neutrophils and mast cells. **Macrophages** and **neutrophils** may act against pathogens by phagocytosis (engulfing in cellular lysosomes and destruction of the pathogen). **Neutrophils** are relatively short lived, act fast and can produce a respiratory burst to destroy the pathogen/microbe. This involves a rapid production of Reactive Oxygen Species (ROS) which may destroy the pathogens. **Macrophages** generally have a longer live span, react slower but more prolonged and may attack via production of nitric oxide and less via ROS. Macrophages produce cytokines to communicate with other members of the immune system, especially cell types of the acquired system. Other members of the innate immune system are mast cells which can excrete e.g. histamine on detection of antigens. Cells of the acquired, or

adaptive immune system mount more specific responses for the immune insult, and are therefore generally more effective. **Lymphocytes** are the cells of the adaptive immune system which can be classified in **B-lymphocytes** and **T-lymphocytes**. **B-lymphocytes** produce antibodies which can serve as cell surface antigen-receptors, essential in the recognition of e.g. microbes. **B-lymphocytes** facilitate humoral (extracellular) immune responses against extracellular microbes (in the respiratory gastrointestinal tract and in the blood/lymph circulation). Upon recognition of an antigen, B-lymphocytes produce species antibodies which bind to the specific antigen. This on the one hand may decrease the infectivity of pathogens (e.g. microbes, viruses) directly, but also mark them for recognition by phagocytic cells. **T-lymphocytes** are active against intracellular pathogens and microbes. Once inside cells, pathogens are out of reach of the B-lymphocytes. **T-lymphocytes** may activate macrophages or neutrophils to destroy phagocytosed pathogens or even destroy infected cells. Both B- and T-lymphocytes are capable of producing an extreme diversity of clones, specific for antigen recognition. Communication between the different immune cells occurs by the production of e.g. cytokines, including interleukins (ILs), chemokines, interferons (IFs), and also Tumour Necrosis Factors (TNFs). Cytokines and TNFs are related to specific responses in the immune system, for instance IL6 is involved in activating B-cells to produce immunoglobulins, while TNF- $\alpha$  is involved in the early onset of inflammation, therefore one of the cytokines inducing acute immune responses. Inflammation is a generic response to pathogens mounted by cells of the innate part of the immune system. It generally results in increased temperature and swelling of the affected tissue, caused by the infiltration of the tissue by leukocytes and other cells of the innate system. A proper acute inflammatory response is not only essential as a first defence but will also facilitate the activation of the adaptive immune system. Communication between immune cells, via cytokines, not only directs cells to the place of infection but also activates for instance cells of the acquired immune system. This is a very short and non-exhaustive description of the immune system, for more details on the functioning of the immune system see for instance Abbas et al. (2018).

Chemicals may affect the immune system in different ways. Exposure to lead for instance may result in immune suppression in waterfowl and raptors (Fairbrother et al. 2004, Vallverdú-Coll et al., 2019). Decreasing spleen weights, lower numbers of white blood cells and reduced ability to mount a humoral response against a specific antigen (e.g. sheep red blood cells), indicated a lower potential of exposed birds to mount proper immune responses upon infection. Exposure to mercury resulted in decreased proliferation of B-cells in zebra finches (*Taeniopygia guttata*), affecting the acquired part of the immune system (Lewis et al., 2013). However, augmentation of the immune system upon exposure to e.g. cadmium has also been reported in for instance small mammals, indicating an enhancement of the immune response (Demenesku et al., 2014). Both immune suppression as well as immune enhancement may have negative impacts on the organisms involved; the former may decrease the ability of the organism to deal with pathogens or other infections, while immune enhancement may increase the energy demands of the organism and it may also result in for instance hypersensitivity or even auto-immunity in organisms.

Chemicals may affect immune cells via **toxicity to mechanisms that are not specific to the immune system**. Since many different cell types are involved in the immune system, the sensitivity to these modes of toxicity may vary considerably among cells and among chemicals. This would imply that as a whole, the immune system may inherently include cells that are sensitive to different chemicals, and as such may be quite sensitive to a range of toxicants. For instance induction of **apoptosis**, programmed cell death, is essential to clear the activated cells involved in an immune response after the infection is minimised and the system is returning to a state of homeostasis (see Figure 2). Chemicals may induce apoptosis, and thus interfere with the kinetics of adaptive immune responses, potentially reducing the longevity of cells.

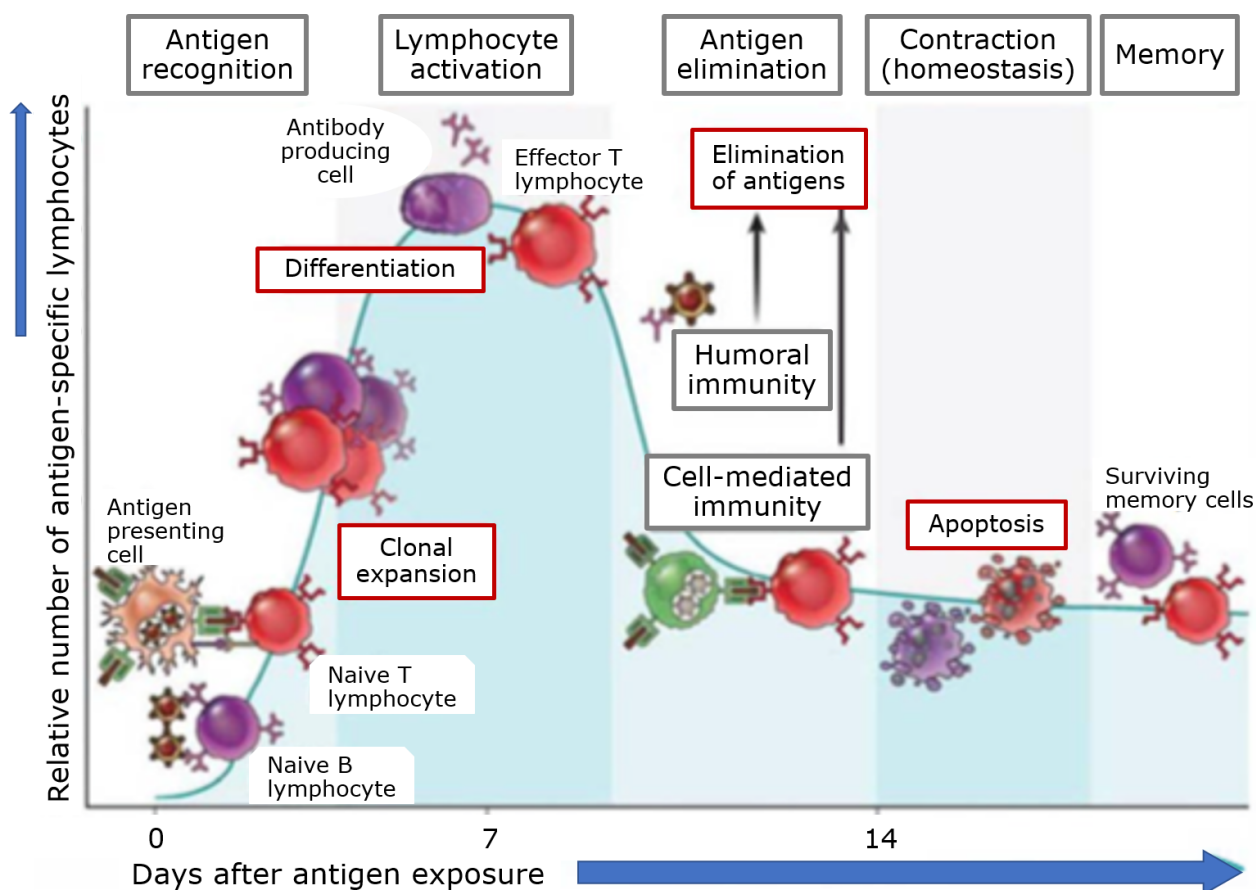


Figure 2. Development of an adaptive immune response, with the different cell types involved. Adapted from [www.memorangapp.com/flashcards/170860/Immunology+Exam+1/](http://www.memorangapp.com/flashcards/170860/Immunology+Exam+1/).

**Toxic effects on mechanisms specific to the immune system** may be related to its functioning. Since the production of ROS and nitric oxides are effector pathways along which neutrophils and macrophages of the innate systems combat pathogens (via a high production of reactive oxygen species, i.e. oxidative burst, to attack pathogens), impacts on the **oxidative status** of these cells may not only result in general toxicity, potentially affecting a range of cell types, but it may also affect the responsiveness of the (innate) immune system particularly. For instance, cadmium has a high affinity to bind to glutathione (GSH), a prominent anti-oxidant in cells, and has shown to affect acute immune responses in thymus and spleens of mice (Pathak and Khandelwal, 2007) via this mechanism. A decrease of GSH by binding of chemicals (like cadmium) may modulate macrophages towards a pro-inflammatory response by changes in the redox status of the cells involved, changing not only their activities against pathogens but potentially also their production and release of cytokines (Dong et al., 1998).

GSH is also involved in the modulation of the acquired immune system by affecting so-called antigen-presenting cells (APCs, e.g. dendritic cells). APCs capture microbial antigens that enter the body, transport these to specific immune-active tissues (e.g. lymph nodes) and present them to naive T-lymphocytes, inducing a proper immune response, so-called T-helper cells. T-helper cells include subsets, e.g. T-helper 1 cells (Th1-cells) and T-helper 2 cells (Th2-cells). Th1 responses are important in the defence against intracellular infections, by activation of macrophages to ingest microbes. Th2-responses may be initiated by infections by organisms too large to be phagocytosed, and mediated by e.g. allergens. As mentioned, GSH depletion may result in changes in cytokine production by APC (Dong et al., 1998), generally affecting the release of Th1-response promoting cytokines. Exposure to chemicals interfering with GSH kinetics may therefore result in a dis-balance between Th1 and Th2 responses and as such affect the responsiveness of the immune system. Cadmium and other metals have a high affinity to bind to GSH and may therefore reduce Th1 responses, while in contrast, GSH promoting chemicals may reduce the organisms' ability to initiate Th2-responses (Pathak & Khandelwal, 2008).

The overview on potential effects that chemicals may have on the immune system as presented here is not exhaustive at all. This is even more complicated because effects may be contextual, meaning that chemicals may have different impacts depending on the situation an organism is in. For instance, the magnitude of immunotoxic effects may be dependent on the general condition of the

organism, and hence some infected animals may show effects from chemical exposure while others may not. Impacts may also differ between types of infection (e.g. Th1 versus Th2 responsive infections). This, together with the complex and dynamic composition of the immune system, limits the development of general dose response relationships and hazard predictions for chemicals. Furthermore, most of the research on effects of chemicals on the immune system is focussed on humans, based on studies on rats and mice. Little is known on differences among species, especially in non-mammalian species which may have completely differentially structured immune systems. Some studies on wildlife have shown effects of trace metals on small mammals (Tersago et al., 2004, Rogival et al., 2006, Tête et al., 2015) and of lead on birds (Vallverdú-Coll et al., 2015). However, specific modes of action are still to be resolved under field conditions. Research on immuno-toxicity in wildlife however is essential not only from a conservational point of view (to protect the organisms and species involved) but also from the perspective of human health. Wildlife plays an important role in the kinetics of zoonotic diseases, for instance small mammals are the prime reservoir for *Borrelia spirochetes*, the causative pathogens of Lyme-disease while migrating waterfowl are indicated to drive the spread of e.g. avian influenza. The role of wildlife in the kinetics of the environmental spread of zoonotic diseases is therefore eminent, which may seriously be affected by chemical induced alterations of their immune system.

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### 4.2.10. Question 1

Name two general reasons why immunomodulation in organisms may be very sensitive to exposure to environmental chemicals

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### 4.2.10. Question 2

Why is it that immunomodulatory chemicals to which humans are not exposed still may have an impact on human health?

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#### 4.2.11. Toxicity mechanisms of metals

**Author:** Nico M. van Straalen

**Reviewers:** Philip S. Rainbow, Henk Schat

##### Learning objectives

You should be able to

- list five biochemical categories of metal toxicity mechanisms and describe an example for each case
- interpret biochemical symptoms of metal toxicity (e.g. functional categories of gene expression profiles) and explain these in terms of the mode of action of a particular metal

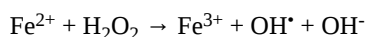
**Keywords:** Reactive oxygen species, protein binding, DNA binding, ion pumps,

##### Synopsis

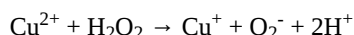
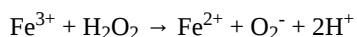
Toxicity of metals on the biochemical level is due to a wide variety of mechanisms, which may be classified as follows, although they are not mutually exclusive: (1) generation of radical oxygen species (Fe, Cu), (2) binding to nucleophilic groups in proteins (Cd, Pb), (3) binding to DNA (Cr, Cd), (4) binding to ion channels or membrane pumps (Pb, Cd), (5) interaction with the function of essential cellular moieties such as phosphate, sulfhydryl groups, iron or calcium (As, Cd, Al, Pb). In addition, these mechanisms may act simultaneously and interact with each other. There are interesting species patterns of susceptibility to metals, e.g. mammals are hardly susceptible to zinc, while plants and crustaceans are. Earthworms, gastropods and fungi are quite sensitive to copper, but not so for terrestrial vertebrates. In this section we discuss five different categories of metal toxicity as well as some patterns of species differences in sensitivity to metals.

##### Generation of reactive oxygen species

Reactive oxygen species (ROS) are activated forms of oxygen that have one or more unpaired electrons in the outer orbit. The best knowns are superoxide anion ( $O_2^-$ ), singlet oxygen ( $^1\Delta gO_2$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^\bullet$ ) (see the section on [Oxidative stress](#)), effective catalyzers of reactive oxygen species. This relates to their capacity to engage in redox reactions with transfer of one electron. One of the most famous reactions is the so-called Fenton reaction catalyzed by reduced iron and copper ions:



Both reactions produce the highly reactive hydroxyl radical ( $OH^\bullet$ ), which may trigger severe cellular damage by peroxidation of membrane lipids (see the section on [Oxidative Stress](#)). Very low concentrations of metal ions can keep this reaction running, because the reduced forms of the metal ions are restored by a second reaction with hydrogen peroxide:



The overall reaction is a metal-catalyzed degradation of hydrogen peroxide, causing superoxide anion and hydroxyl radical as intermediates. Oxidative stress is one of the most important mechanisms of toxicity of metals. This can also be deduced from the metal-induced transcriptome. Gene expression profiling has shown that it is not uncommon that more than 10% of the genome responds to sublethal concentrations of cadmium.

##### Protein binding

Several metals have a great affinity towards sulfhydryl (-SH) groups in the cysteine residues of proteins. Binding to such groups may distort the secondary structure of a protein at sites where SH-groups coordinate to form S-S bridges. The SH-group is a typical example of a nucleophile, that is, a group that easily donates an electron pair to form a chemical bond. The group that accepts the electron pair is called an electrophile. Another amino acid in a protein to which metals are preferentially bound is the imidazole side-chain of histidine. This heterocyclic aromatic group with two nitrogen atoms easily engages into chemical bonds with metal ions. In fact, histidine residues are often used in metalloproteins to coordinate metals at the active site and to transport metals from the roots upwards through the xylem vessels of plants.

A classical case of metal-protein interaction with subsequent toxicity is the case of lead binding to  $\delta$ -aminolevulinic acid dehydratase ( $\delta$ -ALAD). This is an enzyme involved in the synthesis of hemoglobin. It catalyzes the second step in the biosynthetic pathway, the condensation of two molecules of  $\delta$ -aminolevulinic acid to one molecule of porphobilinogen, which is a precursor of porphyrin, a functional unit binding iron in hemoglobin (Figure 1). The enzyme has several sulfhydryl groups that are susceptible to lead. In the erythrocyte more than 80% of lead is in fact bound to the  $\delta$ -ALAD protein (much more than is bound to hemoglobin). Inhibition of  $\delta$ -ALAD leads to decreased porphyrin synthesis, insufficient hemoglobin, loss of oxygen uptake capacity, and eventually anemia.

Because the inhibition of  $\delta$ -ALAD by lead occurs at already very low exposure levels, it makes a very good biomarker for lead exposure. Measurement of  $\delta$ -ALAD activity in blood has been conducted extensively in workers of metal-processing industries and people living in metal-polluted environments. Also in fish, birds and several invertebrates (earthworms, planarians) the  $\delta$ -ALAD assay has been shown to be a useful biomarker of lead exposure. In addition to lead, mercury is known to inhibit  $\delta$ -ALAD, while the inhibitions by both lead and mercury can be alleviated to some extent by zinc.

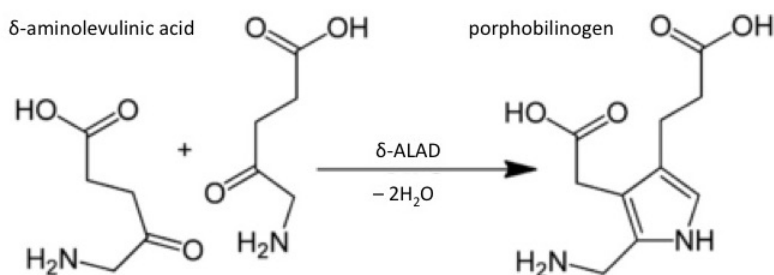


Figure 1. Formation of porphobilinogen from  $\delta$ -ALA, catalyzed by  $\delta$ -ALAD.

## DNA binding

Chromium, especially the trivalent ( $\text{Cr}^{3+}$ ) and the hexavalent ( $\text{Cr}^{6+}$ ) ions are the most notorious metal species known to bind to DNA. Both trivalent and hexavalent chromium may cause mutations and hexavalent chromium is also a known carcinogen. Although the salts of  $\text{Cr}^{6+}$  are only slightly soluble, the reactivity of the  $\text{Cr}^{6+}$ -ion is so pronounced that only very little hexavalent chromium salt is already dangerous.

The genotoxicity of trivalent chromium is due to the formation of crosslinks between proteins and DNA. Any DNA molecule is surrounded by proteins (histones, regulatory proteins, chromatine).  $\text{Cr}^{3+}$  binds to amino acids such as cysteine, histidine and glutamic acid on the one hand, and to the phosphate groups in DNA on the other, without any preference for a specific nucleotide (base). The result is a covalent bond between DNA and a protein that will inhibit transcription or regulatory functions of the DNA segment involved.

Another metal known to interact with DNA is nickel. Although the primary effects of nickel are to induce allergic reactions, it is also a known carcinogen. The exact molecular mechanism is not as well known as in the case of chromium. Nickel could crosslink proteins and DNA in the same way as chromium, but is also argued that nickel's carcinogenicity is due to oxidative stress, resulting in DNA damage. Another suggested mechanism is that nickel could interfere with the DNA repair system.

## Inhibition of ion pumps

Many metals may compete with essential metals during uptake or transport across membranes. A well-known case is the competition between calcium and cadmium at the  $\text{Ca}^{2+}$ ATPase pump in the basal membrane of fish gills (Figure 2).

The gills of fish serve as a target for many water-born toxic compounds because of their large contact area with the water, consisting of several membranes, each with infoldings to increase the surface area, and also their high metabolic activity which stems from their important regulatory activities (uptake of oxygen, uptake of nutrients and osmoregulation). The single-layered epithelium has two types of cells, one active in osmoregulation (called chloride cells), and one active in transport of nutrients and oxygen (called respiratory cells). There are strong tight junctions between these cells to ensure complete impermeability of the epithelium to ions. The apical membrane of the respiratory cells has many uptake pumps and channels (Figure 2). Calcium enters the cells through a calcium channel (without energetic costs, following the gradient). The intracellular calcium concentration is regulated by a calcium-ATPase in the basal membrane, which pumps calcium out of the epithelial cells into the blood.

Figure in preparation



**Figure 2.** Schematic representation of the cells in a fish gill epithelium, showing the fluxes of calcium and cadmium. Calcium enters the cell through calcium channels on the apical side, and is pumped out of the cells into the circulation by a calcium ATPase in the basal membrane. Cadmium ions enter the cells also through the calcium channels, but inhibit the basal calcium ATPase, causing hypocalcemia in the rest of the body. *m* = mucosa (apical side), *s* = serosa (basal side), BP = binding protein, mito = mitochondrion, ER = endoplasmic reticulum. From Verboost et al. (1989).

Water-borne cadmium ions, which resemble calcium ions in their atomic radius, enter the cell through the same apical calcium channels, but subsequently inhibit the basal membrane calcium transporter by direct competition with calcium for the binding site on the ATPase. The consequence is an accumulation of calcium in the respiratory cells, and a lack of calcium in the body of the fish, which causes a variety of secondary effects; amongst others hormonal disturbance, while a severe decline of plasma calcium is a direct cause of mortality. This effect of cadmium occurs at very low concentrations (nanomolar range), and it explains the high toxicity of this metal to fish. Similar cadmium-induced hypocalcemia mechanisms are present in the gill membranes of crustaceans and most likely also in gut epithelium cells of many other species.

## Interaction with essential cellular constituents

There are various cellular ligands outside proteins or DNA that may bind metals. Among these are organic acids (malate, citrate), free amino acids (histidine, cysteine), and glutathione. Metals may also interfere with the cellular functions of phosphate, iron, calcium or zinc, for example by replacing these elements from their normal binding sites in enzymes or other molecules. To illustrate a case of interaction with phosphate we discuss shortly the toxicity of arsenic. Arsenic is strictly speaking not a metal, since arsenic oxide may engage in both base-forming and acid-forming reactions. Together with antimony and four other, lesser-known elements, arsenic is indicated as a "metalloid".

Arsenic is a potent toxicant; arsenic trioxide ( $\text{As}_2\text{O}_3$ ) is well known for its high mammalian toxicity and its use as a rodenticide and wood preservative. There are also therapeutic applications of arsenic trioxide, against certain leukemias and arsenic is often implied in homeopathic treatments. Arsenic compounds are easily transported throughout the body, also across the placental barrier in pregnant women.

Arsenic can occur in two different valency states: arsenate ( $\text{As}^{5+}$ ) and arsenite ( $\text{As}^{3+}$ ). The terms are also used to indicate the oxy-salts, such as ferric arsenate,  $\text{FeAsO}_4$ , and ferric arsenite,  $\text{FeAsO}_3$ . Inside the body, arsenic may be present in oxidized as well as reduced state, depending on the conditions in the cell, and it is enzymatically converted to one or the other state by reductases and oxidases. It may also be methylated by methyltransferases. The two different forms of arsenic have quite different toxicity mechanisms. Arsenate,  $\text{AsO}_4^{3-}$ , is a powerful analog of phosphate, while arsenite ( $\text{AsO}_3^{3-}$ ) reacts with SH-groups in proteins, like the metals discussed above. Arsenite is also a known carcinogen; the mechanism seems not to rely on DNA binding, like in the case of chromium, but on the induction of oxidative stress and interference with cellular signaling.

The most common reason of chronic arsenic poisoning is due to inhibition of the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH). This is a critical enzyme of the glycolysis, converting glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. However, in the presence of arsenate, GAPDH converts glyceraldehyde-3-phosphate into 1-arseno-3-phosphoglycerate. Actually arsenate acts as a phosphate analog to "fool" the enzyme. The product 1-arseno-3-phosphoglycerate does not engage in the next glycolytic reaction, which normally produces one ATP molecule, but it falls back to arsenate and 3-phosphoglycerate, without the production of ATP, while the arsenate released can act again on the enzyme in a cyclical manner. The result is that the glycolytic pathway is uncoupled from ATP-production. Needless to say this signifies a severe and often fatal inhibition of energy metabolism.

## Species patterns of metal susceptibility

Animals, plants, fungi, protists and prokaryotes all differ greatly in their susceptibility to metals. To give a few examples:

- Earthworms and snails are known to be quite sensitive to copper; the absence of earthworms in orchards and vineyards where copper-containing fungicides are used is well documented. Snails cannot be cultured in water that runs through copper-containing linings. Fungi are also sensitive to copper, which explains the use of copper in fungicides. Also many plants are sensitive to copper due to the effects on root growth. Among vertebrates, sheep are quite sensitive to copper, unlike most other mammals.
- Crustaceans as well as fish are relatively sensitive to zinc. Mammals, however, are hardly sensitive to zinc at all.
- Humans are relatively sensitive to lead because high lead exposure lead disturbs the development of children's brain and is correlated with low IQ-scores. Most invertebrates however, are quite insensitive to lead.

- Although many invertebrates are quite sensitive to cadmium, the interspecies variation in sensitivity to this element is particularly high, even within the same phylogenetic lineage. The soil-living oribatid mite *Platynothrus peltifer* is one of the most sensitive invertebrates with respect to the effect of cadmium on reproduction, however *Oppia nitens*, also an oribatid, is extremely tolerant to cadmium.

In the end, such patterns must be explained in terms of the presence of susceptible biochemical targets, different strategies for storage and excretion, and differing mechanisms of defence and sequestration. However, at the moment there is no general framework by which to compare the variation of sensitivity across species. Also, there is no relation between accumulation and susceptibility; some species that accumulate metals to a large degree (e.g. copper in isopods) are not sensitive to the same metal, while others, which do not accumulate the metal, are quite sensitive. Accumulation seems to be partly related to a species feeding strategy (e.g. spiders absorb almost all the (fluid) food they take in and any metals in the food will accumulate in the midgut gland); accumulation is also related to specific nutrient requirements (e.g. copper in isopods, manganese in some oribatid mites). Finally, some populations of some species have evolved specific tolerances in response to their living in a metal-contaminated environment, on top of the already existing accumulation and detoxification strategies.

## Conclusion

Metals do not form a homogeneous group. Their toxicity involves reactivity towards a great variety of biochemical targets. Often several mechanisms act simultaneously and interact with each other. Induction of oxidative stress is a common denominator, as is reaction to nucleophilic groups in macromolecules. The great variety of metal-induced responses makes them interesting model compounds for toxicological studies.

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### 4.2.11. Question 1

Mention three different classes of primary lesions due to free metal ions and causing metal toxicity. Include the metals that are best known for causing each type of lesion.

### 4.2.11. Question 2

Is it possible to decide to which type of metal a cell is exposed, based on the kind of cellular disturbance that is observed?

### 4.2.11. Question 3

Several invertebrate accumulate metals to a very high degree. Mention a few examples and the metals they accumulate. Are these animals also among the most sensitive to metal toxicity? Please explain.

### 4.2.11. Question 4

"Essential metals can be regulated and are therefore less toxic than xenobiotic metals" - Please comment on this thesis.

## 4.2.12. Metal tolerance

**Author:** Nico M. van Straalen

**Reviewers:** Henk Schat, Jaco Vangronsveld

### Learning objectives

You should be able to

- describe which mechanisms of changes in metal trafficking can contribute to metal tolerance and hyperaccumulation
- explain the molecular factors associated with the evolution of metal tolerance in plants and in animals

- develop an opinion on the issue of "rescue from pollution by evolution" in the risk assessment of heavy metals

**Keywords:** hyperaccumulation, metal uptake mechanisms, microevolution

## Synopsis

Some species of plants and animals have evolved metal-tolerant populations that can survive exposures that are lethal for other populations of the same species. Best known is the heavy metal vegetation that grows on metalliferous soils. The study of these cases of "evolution in action" has revealed many aspects of metal trafficking in plants, transport across membranes, metal scavenging molecules in the cell, and subcellular distribution of metals, and how these processes have been adapted by natural selection for tolerance. Metal-tolerant plant varieties are usually dependent upon high metal concentrations in the soil and do not grow well in reference soils. In addition, some plant species show an extreme degree of metal accumulation. In animals metal tolerance has been demonstrated in some invertebrates that live in close contact with metal-containing soils and this is usually achieved by altered regulation of metal scavenging proteins such as metallothioneins, or by duplication of the corresponding genes. Genomics studies are broadening our perspective as the adaptation normally does not rely on a single gene but includes hypostatic factors and modifiers.

## Introduction

As metals cannot be degraded or metabolized, the only way to deal with potentially toxic excess is to store or excrete them. Often both mechanisms are operational, excretion being preceded by storage or scavenging, but animals and plants differ greatly in the emphasis on one or the other mechanism. Both essential and nonessential metals are subject to all kinds of trafficking mechanisms aiming to keep the biologically active, free ion concentration of the metal extremely low. Still, there is hardly any relationship between accumulation and tolerance. Some species have low tissue concentrations and are sensitive, others have low tissue concentrations and are tolerant, some accumulate metals and suffer from the high concentrations, others accumulate and are extremely tolerant.

Like the mechanisms of biotransformation (see the section on [Genetic Variation](#)) metal trafficking mechanisms show genetic variation and such variation may be subject to evolution. However, it has to be noted that only in a limited number of plants and animal species metal-tolerant populations have evolved. This may be due to the fact that evolution of metal tolerance makes use of already existing, moderately efficient, metal trafficking mechanisms in the ancestral species. This interpretation is suggested by the observation that the non-metal-tolerant varieties of metal-tolerant plants already have a certain degree of metal tolerance (larger than species that never evolve metal-tolerant varieties). So the mutational distance to metal tolerance was smaller in the ancestors of metal-tolerant plants than it is in "normal" plants.

Real metal tolerance, where the metal-tolerant population can withstand orders of magnitude larger exposures than reference populations, and has become dependent on metal-rich soils, is only found in plants. Metal tolerance in animals is of degree, rather than of kind, and does not come with externally recognizable phenotypes. Most likely the combination of strong selection pressure, the impossibility to escape by locomotion and the right pre-existing genetic variation explain why metal tolerance in plants is so much more prominent compared to animals.

In this section we will discuss the various mechanisms that have been shown to underlie metal tolerance. The evolutionary response to environmental metal exposure is one of the classical examples of "evolution in action", next to insecticide resistance in mosquitoes and industrial melanism in butterflies.

### Metal tolerance in plants

For many years, most likely already since humans started to dig ores and use metals for the manufacture of utensils, pottery and tools, it has been known that naturally metal-rich soils harbour a specific metal-tolerant vegetation. This "Schwermetallvegetation", described in the classical book by the German-Dutch botanist W.H.O. Ernst, consists of a designated collection of plant species, with representatives from various families. Several species also have metal-sensitive populations living in normal soils, but some, like the European zinc violet, *Viola calaminaria*, are restricted to metal-rich soils. This is also seen in the metal-tolerant vegetations of New Caledonia, Cuba, Zimbabwe and Congo, which to a large degree consist of endemic metal-tolerant species (true metallophytes) that are never found in normal soils. However, some common species also developed metal-tolerant ecotypes.

Metal-tolerant plant species have expanded their range when humans started to dig the metal ores and now can also be found extensively at mining sites, metal-enriched stream banks, and around metal smelters. Naturally metal-enriched soils differ from

reference soils not only in metal concentration but also in other aspects, e.g. calcium and moisture, so the selection for metal tolerance comes goes hand-in-hand with selection by several other factors.

Metal tolerance is mainly restricted to herbs and forbs, and (except some tropical serpentine) does not extend to trees. A heavy metal vegetation is recognizable in the landscape as a "meadow", lacking trees, with relatively few plant species and an abundance of metallophytes. In the past, metal ores were discovered from the presence of such metallophytes, an activity called bioprospecting.

We know from biochemistry that different metals are bound to different ligands and follow different biochemical pathways in biological tissues (see the section on [metal accumulation](#)). Some metals (cadmium, copper, mercury) are "sulphur-seekers", others have an affinity to organic acids (zinc) and still others tend to be associated with calcium-rich tissues (lead). Essential metals such as copper, zinc and iron have their own, metal-specific, transport mechanisms. From these observations one may conclude that metal tolerance will also be specific to the metal and that cross-tolerance (tolerance directed to one metal causing tolerance to another metal as a side-effect) is relatively rare. This is indeed the case.

In many cases metal-tolerant plants do not show the same growth characteristics as the non-tolerant varieties of the same species. Loss of growth potential has often been interpreted as a "cost of tolerance". However, genetic research has shown that the lower growth potential of metallophytes is a separate adaptation, to deal with the usually infertile metalliferous soils, and there is no mechanistic link to tolerance. Metabolic costs or negative pleiotropic effects of metal tolerance have not been described. The fact that metal-tolerant plants do not grow well in clean soils is explained by the constitutive upregulation of trafficking and compartmentalization mechanisms, causing increased metal requirements that cannot be met on non-metalliferous soils.

Another striking fact is that metal tolerances in the same plant species at different sites have evolved independently from each other. The various metal-tolerant populations of a species do not all descend from a single ancestral population, but result from repeated local evolution. That still in different populations sometimes the same loci are affected by natural selection, is ascribed to the fact that, given the species' genetic background, there are only a limited number of avenues to metal tolerance.

A final general principle is that metal tolerance in plants is often targeted towards proteins that transport metals across membranes (cell membrane, tonoplast). The genes of such transporters may be duplicated, the balance between high-affinity transporters and low-affinity versions may be altered, their expression may be upregulated or downregulated, or the proteins may be targeted to different cellular compartments.

Although many details on the genetic changes responsible for tolerance in plants are still lacking, the work on copper tolerance in bladder campion, *Silene vulgaris*, illustrates many of the points listed above. The plant has many metal-tolerant populations, of which one found at Imsbach, Germany, shows an extreme degree of copper tolerance and also some (independently evolved) zinc and cadmium tolerance. The area is known for its "Bergbau" with historical mining activities for copper, silver and cobalt, but also some older calamine deposits, which explains the zinc and cadmium tolerance.

Genetic work by H. Schat and colleagues has shown that two ATP-driven copper transporters, designated *HMA5I* and *HMA5II* are involved in copper tolerance of *Silene*. The *HMA5I* protein resides in the tonoplast to relocate copper into the vacuole, while *HMA5II* resides in the endoplasmic reticulum. When free copper ions appear in the cell, *HMA5II* relocates from the ER to the cell membrane and starts pumping copper out of the cell. During transport from roots to shoot (in the xylem vessels) copper is bound as a nicotianamine complex. In addition, plant metallothioneins play a role in copper binding and transport in the phloem and during redistribution from senescent leaves. Copper tolerance in *Silene* illustrates the principle referred to above that metal tolerance is achieved by enhancing the transport mechanisms already present, not by evolving new genes.

## Metal hyperaccumulation

Some plants accumulate metals to an extreme degree. Well-known are metallophytes growing on serpentine soils, which accumulate very large amounts of nickel. Also copper and cobalt accumulation is observed in several species of plants. Hyperaccumulators do not exclude metals but preferentially accumulate them when the concentration in the soil is extremely high (> 50.000 mg of copper per kg soil). The copper concentration of the leaves may reach values of more than 1000 µg/g. A very extreme example is a tree species, *Sebertia acuminata*, growing on the island of New Caledonia in ultramafic soil with 0.85% of nickel, which produces a latex containing 11% of nickel by weight. Such extraordinary high concentrations impose extreme demands on the efficiency of metal trafficking and so have attracted the attention of biological investigators. In Western Europe's heavy metal vegetation, zinc accumulators are present in several species of the genera *Agrostis*, *Brassica*, *Thlaspi* and *Silene*.

Most of the experimental research is conducted on the brassicacean species *Noccaea (Thlaspi) caerulescens* and *Arabidopsis halleri*, with *Arabidopsis thaliana* as a non-accumulating reference model.

The transport of metals in a plant involves a number of distinct steps, where each step is upregulated in the metal hyperaccumulator. This illustrated in Figure 1 in Verbruggen et al. (2009) for zinc hyperaccumulation in *Thlaspi caerulescens*.

- Uptake in root epithelial cells; this involves ZIP4 and IRT1 zinc transporters
- Transport between root tissues
- Loading of the root xylem, by means of HMA4 and other metal transporters
- In the xylem zinc may be chelated by citrate, histidine or nicotianamine, or may just be present as free ions
- Unloading of the xylem in the leaves. This involves YSL proteins and others.
- Transport into vacuoles and chelation to vacuole-specific chelators such as malate, involving metal transporters such as HMA3, MTP1 and MHX.

While the basic components of the system are beginning to be known, the question how the whole machinery is upregulated in a coherent fashion is not yet clear.

### Metal tolerance in animals

Also in animals, metal tolerant populations of the same species have been reported, however, there is no specific metal-tolerant community with a designated set of species, like in plants. There are, however, obvious metal accumulators among animals. Best known are terrestrial isopods, which accumulate very high concentrations of copper in designated cells in the their hepatopancreas, and some species of oribatid mites which accumulate very high amounts of manganese and zinc.

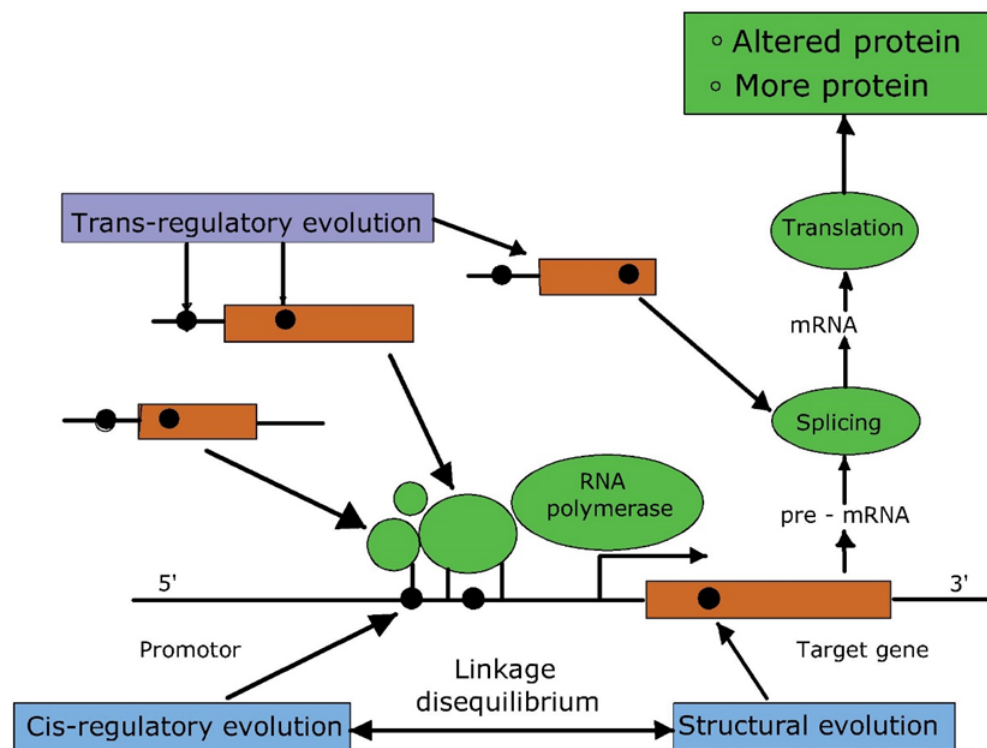


Figure 2. Simplified scheme of transcriptional regulation of a gene involved in metal detoxification, such as metallothionein. The dots indicate the various mutations possible. Two different pathways to tolerance are sketched: structural mutations altering the protein (e.g. increasing binding affinity) and regulatory mutations altering the amount of protein by regulating transcription. Transcriptional regulation can be in cis (changes in the promoter, affecting the binding of transcription factors) or in trans (transcription factor or other regulatory proteins). Redrawn from Van Straalen et al. (2011) by Wilma IJzerman.

One of the factors investigated to explain metal tolerance in animals is a metal-binding protein, metallothionein (MT). Gene duplication of an MT gene has been implicated in the tolerance of *Daphnia* and *Drosophila* to copper. In addition, metal tolerance may be due to altered transcriptional regulation. The latter mechanism underlies the evolution of cadmium tolerance in the soil-living springtail, *Orchesella cincta*. Detailed genetic analysis of this model system has revealed that the MT promoter of *O. cincta*

shows a very large degree of polymorphism, some alleles affecting the transcription factor binding sites and causing overexpression of MT. The promoter allele conferring strong overexpression of MT upon exposure to cadmium, had a significantly higher frequency in *O. cincta* populations from metal-contaminated soils (Figure 2).

In addition to springtails, evolution of metal tolerance has also been described for the earthworm, *Lumbricus rubellus*. In a population living in a lead-contaminated deserted mining area in Wales two lineages were distinguished on the basis of the COI gene and RFLPs. Interestingly, the two lineages had colonized different microhabitats of the area, one of them being unable to survive high lead concentrations. Differential expressions were noted for genes in phosphate and calcium metabolism. Two crucial mutations in a calcium transport protein suggested that lead tolerance in *L. rubellus* is due to modification of calcium transport, a logical target since lead and calcium are often found to interact with each other's transport (see the section on [metal accumulation](#)).

## Conclusions

The study of metal tolerance is a rewarding topic of evolutionary ecotoxicology. Several crucial genetic mechanisms have been identified but in none of the study systems a complete picture of the evolved tolerance mechanisms is available. It may be expected that genome-wide studies will be able to identify the full network responsible for tolerance, which most likely includes not only major genes, but also hypostatic factors and modifiers.

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### 4.2.12. Question 1

Describe the pathway of zinc ions taken up by plants from the soil solution to their final destination in the plant, and how the various steps have been modified in hyperaccumulation plants species such as *Arabidopsis halleri*.

### 4.2.12. Question 2

Discuss the difference between trans-regulatory change and cis-regulatory change in the evolution of metal tolerance.

### 4.2.13. Adverse Outcome Pathways

**Author:** Dick Roelofs

**Reviewers:** Nico van Straalen, Dries Knapen

#### Learning objectives:

You should be able to

- explain the concept of adverse outcome pathway.
- interpret a graphical representation of an AOP



- search the AOPwiki database for molecular initiating events, key events and adverse outcomes

**Keywords:** Molecular initiation event, key event, in vitro assay, high throughput assay, pathway

## Introduction

Over the past two decades the availability of molecular, biochemical and genomics data has exponentially increased. Data are now available for a phylogenetically broad range of living organisms, from prokaryotes to humans. This has tremendously advanced our knowledge and mechanistic understanding of biological systems, which is highly beneficial for different fields of biological research such as genetics, evolutionary biology and agricultural sciences. Being an applied biological science, toxicology has not yet tapped this wealth of data, because it is difficult to incorporate mechanistic data when assessing chemical safety in relation to human health and the environment. However, society is increasingly concerned about the release of industrial chemicals with little or no hazard- or risk information. Consequently, a much larger number of chemicals need to be considered for potential adverse effects on human health and ecosystem functioning. To meet this challenge it is necessary to deploy fast, cost-effective and high throughput approaches that can predict potential toxicity of substances and replace traditional tests based on survival and reproduction that run for weeks or months and often are quite labour-intensive. A major challenge is however, to link these fast *in vitro* and *in vivo* assays to endpoints used in current risk assessment. This challenge was picked up by defining the adverse outcome pathway (AOP) framework, for the first time proposed by the Gerald Ankley and co-workers from the United States Environmental Protection Agency, US-EPA (Ankley et al., 2010).

## The framework

The AOP framework is defined as an evolution of prior pathway-based concepts, most notably mechanisms and modes of action, for assembling and depicting toxicological data across biological levels of organization (Ankley and Edwards, 2018). An AOP is a graphical representation of a series of measurable *key events* (KEs). A key event is a measurable directional change in the state of a biological process. KEs can be linked to one another through *key event relationships* (KERs; see Figure 1). The first KE is depicted as the "*molecular initiating event*" (MIE), and represents the interaction of the chemical with a biological receptor that activates subsequent key events. The key event relationships should ideally be based on causal evidence. A cascade of key events can eventually result in an *adverse outcome* (AO) at the individual or population level. The MIE and AO are specialized KEs, but treated like any other KE in the AOP framework.

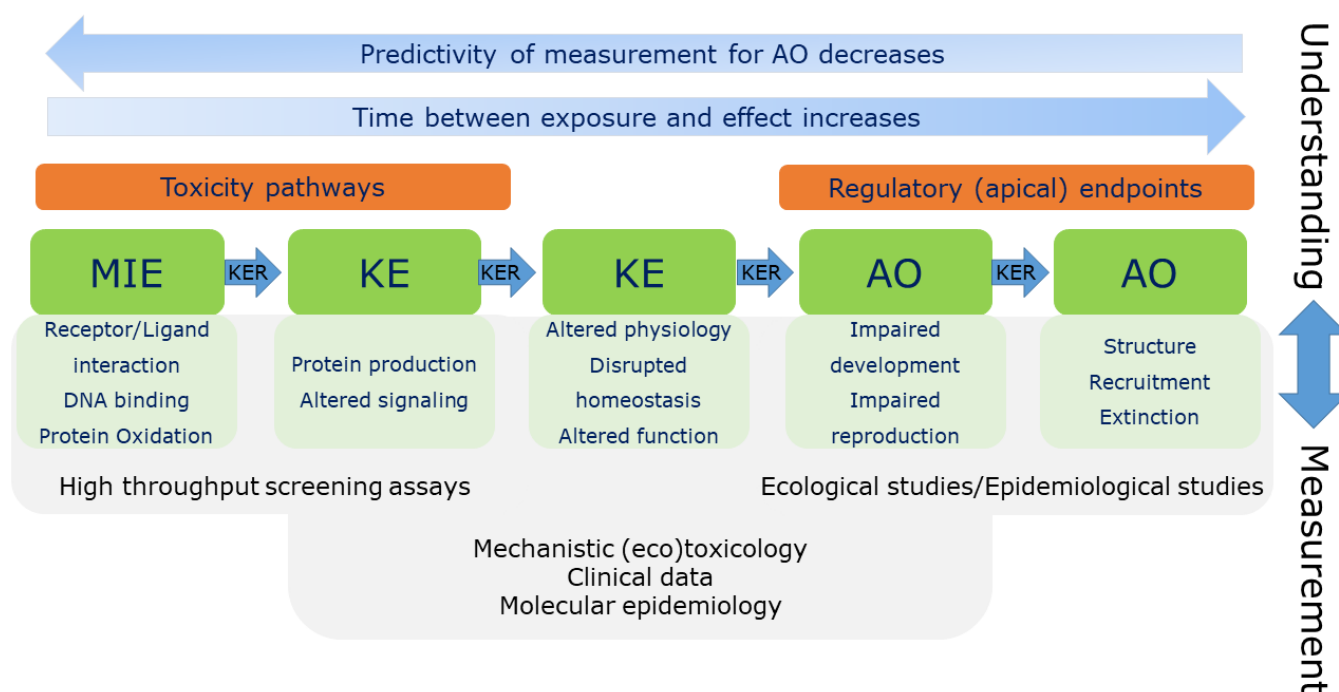


Figure 1. The Adverse Outcome Pathway framework. Various measurable data streams are linked in a causal manner and eventually are related to outcomes essential for risk assessment. MIE, molecular initiating event; KE, key event; KER, key event relationship; AO, adverse outcome. Adapted from Ankley and Edwards (2018) by Kees van Gestel.

The aim of an AOP is to represent and describe, in a simplified way, how responses at the molecular- and cellular level are translated to impacts on development, reproduction and survival, which are relevant endpoints in risk assessment (Villeneuve et al., 2014). Five core concepts have been defined in the development of AOPs:

1. AOPs are not chemical specific, they are biological pathways;
2. AOPs are modular, they refer to a designated and defined metabolic cascade, even if that cascade interacts with other biological processes;
3. individual AOPs are developed as pragmatic units;
4. networks of multiple AOPs sharing KEs and KERs are functional units of prediction for real-world scenarios; and
5. AOPs are living documents that may change over time based on new scientific insights.

Generally, AOPs are simplified linear pathways but different AOPs can be organized in networks with shared nodes. The AOP networks are actually the functional units of prediction, because they represent the complex biological interactions that occur in response to exposure to a toxicant or a mixture of toxicants. Analysis of the intersections (shared key events) of different AOPs making up a network can reveal unexpected biological connections (Villeneuve et al., 2014).

## Molecular initiating events and key events

Typically, an AOP consists of only one MIE, and one AO, connected to each other by a potentially unlimited number of KEs and KERs. The MIE is considered to be the first anchor of an AOP at the molecular level, where stressors directly interact with the biological receptor. Identification of the MIE mostly relies on chemical analysis, *in silico* analysis or *in chemico* and *in vitro* data. For instance, the MIE for AOPs related to estrogen receptor activation involves the binding of chemicals to the estrogen receptor, thereby triggering a cascade of effects in hormone-related metabolism (see the section on [Endocrine disruption](#)). The MIE for AOPs related to skin sensitization (see below) involves the covalent interaction of chemicals to skin proteins in skin cells, an event called haptenization (Vinken, 2013).

A wide range of biological data can support the understanding of KEs. Usually, early KEs (directly linked to MIEs) are assessed using *in vitro* assays, but may include *in vivo* data at the cellular level, while intermediate and late KEs rely on tissue-, organ- or whole organism measurements (Figure 1). Key-event measurements are also related to data from high-throughput screening and/or data generated by different -omics technologies. This is actually where the true value of the AOP framework comes in, since it is



currently the only framework able to reach such a high level of data integration in the context of risk assessment. It is even possible to integrate comparative data from phylogenetically divergent organisms into key event measurements, valid across species, which could facilitate the evaluation of species sensitivity (Lalone et al., 2018). The final AO output is usually represented by apical responses, already described as standard guidelines accepted and instrumental in regulatory decision-making, which include endpoints such as development, growth, reproduction and survival.

Development of the AOP framework is currently supported by US-Environmental Protection Agency, EU Joint Research Centers (ERC) and the Organization for Economic Cooperation and Development (OECD). Moreover, OECD has sponsored the development of an open access searchable database AOPWiki (<https://aopwiki.org/>), comprising over 250 AOPs with associated MIEs, KEs and KERs, and more than 400 stressors. New AOPs are added regularly. The database also has a system for specifying the confidence to be placed in an AOP. Where KEs and KERs are supported by direct, specifically designed, experimental evidence, high confidence is placed in them. In other cases confidence is considered moderate or low, e.g. when there is a lack of supporting data or conflicting evidence.

### Case example: covalent protein binding leading to skin sensitization (AOP40)

Skin sensitization is characterized by a two-step process, a sensitization phase and an elicitation phase. The first contact of electrophile compounds with the skin covalently modifies skin proteins and generates an immunological memory due to generated antigen/allergen specific T-cells. During the elicitation phase, repeated contact with the compound elicits the allergic reaction defined as allergic contact dermatitis, which usually develops into a lifelong effect. This is an important endpoint for safety assessment of personal care products, traditionally evaluated by *in vivo* assays. Based on changed public opinion the European Chemical Agency (ECHA) decided to move away from whole animal skin tests, and developed alternative assessment strategies. During sensitization, the MIE takes place when the chemical enters the skin, where it forms a stable complex with skin-specific carrier proteins (hapten complexes), which are immunogenic. A subsequent KE comprises inflammation and oxidative defense via a signaling cascade called the Keap1/Nrf2 signalling pathway (*Kelch-like ECH-associated protein 1 / nuclear factor erythroid 2 related factor 2*). At the same time, a second KE is defined as dendritic cell activation and maturation. This results into movement of dendritic cells to lymph nodes, where the hapten complex is presented to naive T-cells. The third KE describes the proliferation of hapten-specific T-cells and subsequent movement of antigen-specific memory cells that circulate in the body. Upon a second contact with the compound, these memory T-cells secrete cytokines that cause an inflammation reaction leading to the AO including red rash, blisters, and burning skin (Vinken et al., 2017). This AOP is designated AOP40 in the database of adverse outcome pathways.

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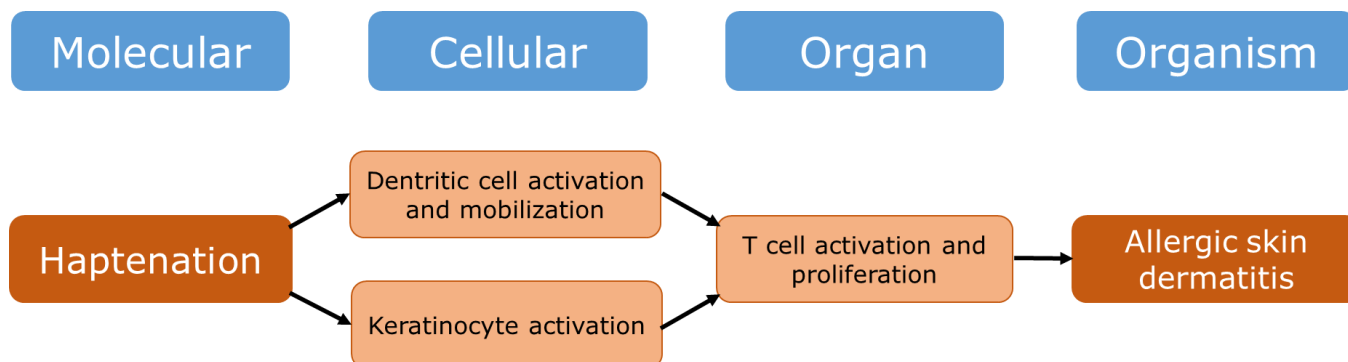


Figure 2. Adverse Outcome Pathway for covalent protein binding leading to skin sensitization (AOP40). Adapted from Vinken et al. (2017) by Kees van Gestel.

A suite of high throughput *in vitro* assays have now been developed to quantify the intermediate KEs in AOP40. These data formed the basis for the development of a Bayesian network analysis that can predict the potential for skin sensitization. This example highlights the use of pathway-derived data organized in an AOP, ultimately leading to an alternative fast screening method that may replace a conventional method using animal experiments.

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#### 4.2.13. Question 1

What is a molecular initiation event?

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#### 4.2.13. Question 2

Where in the chain of events do high-throughput *in-vitro* assays feed into AOPs?

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#### 4.2.13. Question 3

What is the ultimate goal of the AOP concept?

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#### 4.2.13. Question 4

How many intermediate key events are captured in the AOP for skin sensitization?

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#### 4.2.13. Question 5

Is an AOP always represented as a linear chain between MIE and AO?

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#### 4.2.14. Genetic variation in toxicant metabolism

**Author:** Nico M van Straalen

**Reviewers:** Andrew Whitehead, Frank van Belleghem

##### **Learning objectives:**

You should be able to

- explain four different classes of CYP gene variation and expression, contributing to species differences
- explain the associations between biotransformation activity and specific ecologies
- explain how genetic variation in biotransformation enzymes may lead to evolution of toxicant tolerance
- describe the relevance of human genetic polymorphisms for personalized medicine

**Keywords:** toxicant susceptibility; genetic variation; biotransformation evolution of toxicant tolerance

##### **Assumed prior knowledge and related modules**

- Biotransformation and internal processing of chemicals
- Defence mechanisms
- Genetic erosion

In addition, a basic knowledge of genetics and evolutionary biology is needed to understand this module.

##### **Synopsis**

Susceptibility to toxicants often shows inter-individual differences associated with genetic variation. While such differences are considered a nuisance in laboratory toxicity testing, they are an inextricable aspect of toxicant effects in the environment. Variation may be due to polymorphisms in the target site of toxicant action, but more often differences in metabolic enzymes and rates of

excretion contribute to inter-individual variation. The structure of genes encoding metabolic enzymes, as well as polymorphisms in promoter regions of such genes are common sources of genetic variation. Under strong selection pressure species may evolve toxicant-tolerant populations, for example insects to insecticides and bacteria to antibiotics. In human populations, polymorphisms in drug metabolizing enzymes are mapped to provide a basis for personal therapies. This module aims to illustrate some of the genetic principles explaining inter-individual variation of toxicant susceptibility and its evolutionary consequences.

## Introduction

For a long time it has been known that human subjects may differ markedly in their responses to drugs: while some patients hardly respond to a certain dosage, others react vehemently. Similar differences exist between the sexes and between ethnic groups. To avoid failure of treatment on the one hand and overdosing on the other, such personal differences have attracted the interest of pharmacological scientists. Also the tendency to develop cancer upon exposure to mutagenic chemicals is partly due to genetics. Since the rise of molecular ecology in the 1990s ecotoxicologists have noted that inter-individual differences in toxicant responses also exists in the environment.

Due to genetic variation environmental pollution may trigger evolutionary change in the wild. From quantitative genetics we know that when a trait is due to many genes, each with an independent additive effect on the trait value, the response to selection  $R$ , is linearly related to the selection differential  $S$  according to the formula:  $R = h^2 S$ , where  $h^2$  is a measure of the heritability of the selected trait (fraction of additive genetic variance relative to total phenotypic variance). Since anthropogenic toxicants can act as very strong selective agents (large  $S$ ) it is expected that whenever  $h^2 > 0$  there will be adaptation. However, the effectiveness of "evolutionary rescue" from pollution is limited to those species that have the appropriate genetic variation and the ability to quickly increase in population size.

## Polymorphisms of drug metabolizing enzymes in humans

One of the most important enzyme systems contributing to metabolism of xenobiotic chemicals is the cytochrome P450 family, a class of proteins located in the smooth endoplasmic reticulum of the cell and acting in co-operation with several other proteins. Cytochrome P450 will oxidize the substrate and enhance its water-solubility (called phase-I reaction), and in many cases activate it for further reactions involving conjugation with an endogenous compound (phase II reactions). These processes generally lead to detoxification and increased excretion of toxic substances. The biochemistry of drug metabolism is discussed in detail in the section on [Xenobiotic metabolism and defence](#).

The human genome has 57 genes encoding a P450 protein. The genes are commonly designated as "CYP". Other organisms, especially insects and plants have many more CYPs. For example, the *Drosophila* genome encodes 83 functional P450 genes and the genome of the model plant *Arabidopsis* has 244 CYPs. Based on sequence similarity, CYPs are classified in 18 families and 43 subfamilies, but there is no agreement yet about the position of various CYP genes in lower invertebrates. The complexity is enhanced by duplications specific to certain evolutionary lineages, creating a complicated pattern of orthologs (homologs by descent from a common ancestor) and paralogs (homologs due to duplication in the same genome). In addition to functional enzymes it is also common to find many CYP pseudogenes in a genome. Pseudogenes are DNA-sequences that resemble functional genes, but are mutated and they do not result in functional proteins).

The expression of CYP enzymes is markedly tissue-specific. Often CYP expression is high in epithelial tissues (lung, intestine) and organs with designated metabolic activity (liver, kidney). In the human body, the liver is the main metabolic organ and is known for its extensive CYP expression. P450 enzymes also differ in their inducibility by classes of chemicals and in their substrate specificity.

It is often assumed that the versatility of an organism's CYP genes is a reflection of its ecology. For example, herbivorous insects that consume plants of different kinds with many different feeding repellents must avail of a wide diversity of CYP genes. It has also been shown that activity of CYP enzymes among terrestrial organisms is, in general, higher than among aquatic organisms and that plant-eating birds have higher biotransformation activities than predatory birds.

One of the best-investigated CYP genes, especially due to its strong inducibility and involvement in xenobiotic metabolism, is mammalian CYP1A1. In humans induction of this gene is associated with increased lung cancer risk from smoking, and with other cancers, such as breast cancer and prostate cancer. Human CYP1A1 is located on chromosome 15 and encodes 251 amino acids in seven exons (see [Figure 2 in Zhou et al., 2009](#)). About 133 single-nucleotide polymorphisms (SNPs, variations in a single nucleotide that occur at a specific position in the genome) have been described for this gene, of which 23 are non-synonymous (causing a substitution of an amino acid in the protein).

Many of these SNPs have a medical relevance. For example, a rather common SNP in exon 7 changes codon 462 from isoleucine into valine. The substituted allele is called CYP1A1\*2A, and this occurs at a frequency of 19% in the Caucasian part of the human population. The allelic variant of the enzyme has a higher activity towards 17 $\beta$ -estradiol and is a risk factor for several types of cancer. However, the expression of such traits may vary from one population to another, and may also interact with other risk factors. For example, CYP1A1\*2A is a risk factor for cervical cancer in women with a history of smoking in the Polish population, but the same SNP may not be a risk factor in another population or among people with a non-smoking lifestyle. In genetics these effects are known as epistasis: the phenotypic effect of genetic variation at one locus depends on the genotype of another locus. This is also an example of a genotype-by-environment interaction, where the phenotypic effect of a genetic variant depends on the environment (smoking habit). In toxicology it is known that polymorphisms of phase II biotransformation enzymes may significantly contribute to epistatic interaction with CYP genes. Unraveling all these complicated interactions is a very active field of research in human medical genetics.

## Cytochrome P450 variation across species

Comparison of CYP genes in different species has revealed an enormously rapid evolution of this gene family, with many lineage-specific duplications. This indicates strong selective pressures imposed by the need to detoxify substances ingested with the diet. Especially herbivorous animals are constantly exposed to such compounds, synthesized by plants to deter feeding. We also see profound changes in CYP genes associated with evolutionary transitions such as colonization of terrestrial habitats by the various lineages of arthropods. Such natural variation, induced by plant toxins and habitat requirements, is also relevant in the responses to toxicants.

In general, variation of biotransformation enzymes can be classified in four main categories

1. Variation in the structure of the genes, e.g. substitutions that alter the binding affinity to substrates; such variation discriminates the various CYP genes.
2. Copy number variation; duplication usually leads to an increase in enzymatic capacity; this process has been enormously important in CYP evolution. Because CYP gene duplications are often specific to the evolutionary lineage, a complicated pattern of paralogs (duplicates within the same genome) and orthologs (genes common by descent, shared with other species) arises.
3. Promoter variation, e.g. due to insertion of transposons or changes in the number or arrangement of transcription factor binding sites. This changes the amount of protein produced from one gene copy by altered transcriptional regulation.
4. Variation in the structure, action or activation of transcriptional regulators. The transcription of biotransformation enzymes is usually induced by a signaling pathway activated by the compound to be metabolized (see the section on [Xenobiotic metabolism and defence](#)), and this pathway may show genetic variation.

To illustrate the complicated evolution of biotransformation genes, we shortly discuss the CYPs of common cormorant, *Phalacrocorax carbo*. This is a bird known for its narrow diet (fish) and extraordinary potential for accumulation of dioxin-related compounds (PCBs, PCDDs and PCDFs). Environmental toxicologists have identified two CYP1A genes in the cormorant, called CYP1A4 and CYP1A5. It turns out that CYP1A4 is homologous by descent (orthologous) to mammalian CYP1A1 while CYP1A5 is an ortholog of mammalian CYP1A2. However, the orthologies are not revealed by common phylogenetic analysis if the whole coding sequence is used in the alignment (see Figure 3 in Kubota et al., 2006). This is a consequence of a process called interparalog gene conversion, which tends to homogenize DNA sequences of gene copies located on the same chromosome. This diminishes sequence variation between the paralogs, and creates chimeric gene structures, that are more similar to each other than expected from their phylogenetic relations. If a phylogenetic tree is made using a section of the gene that remained outside the gene conversion, the true phylogenetic relations are revealed (see Figure 3 in Kubota et al., 2006).

## Cytochrome P450-mediated resistances

Cytochrome P450 polymorphisms are also implicated in certain types of insecticide resistance. There are many ways in which insects and other arthropods can become resistant and several mechanisms may even be present in the same resistant strain. Target site alteration (making the target less susceptible to the insecticide, e.g. altered acetylcholinesterase, substitutions in the GABA-receptor, etc.) seems to be the most likely mechanism for resistance, however, such changes often come with substantial costs as they may diminish the natural function of the target (in genetics this is called pleiotropy). Increased metabolism does not usually contribute metabolic costs and this is where cytochromes P450 come into play. A model system for investigating the genetics of such mechanisms is DDT resistance in the fruit fly, *Drosophila melanogaster*.

In a DDT-resistant *Drosophila* strain, all CYP genes were screened for enhanced expression and it was shown that DDT resistance was due to a highly upregulated variant of only a single gene, Cyp6g1. Further analysis showed that the gene's promoter carried an insertion with strong similarity to a transposable element of the *Accord* family. The insertion of this element causes a significant overexpression and a high rate of protein synthesis that allows the fly to quickly degrade a DDT dose. The fact that a simple change, in only one allele, can underlie such a distinctive phenotype as pesticide resistance is a remarkable lesson for molecular toxicology.

A recent study on killifish, *Fundulus heteroclitus*, along the East coast of the United States has revealed a much more complicated pattern of resistance. Populations of these fish live in estuaries, some with severely polluted sediments, containing high concentrations of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). Killifish from the polluted environments are much more resistant to toxicity from the model compounds PCB126 and benzo(a)pyrene. This resistance is related to mutations in the gene encoding aryl hydrocarbon receptor (AHR), the protein that binds PAHs and certain PCB metabolites and activates CYP expression. Also mutations in a protein called aryl-hydrocarbon receptor-interacting protein (AIP), a protein that combines with AHR to ensure binding of the ligand, contribute to down-regulation of the CYP1A1 pathway. The net result is that killifish CYP1A1 shows only moderate induction by PCBs and PAHs and the damaging effects of reactive metabolites are avoided. However, since direct knockdown of CYP1A1 does not provide resistance it is still unclear whether the beneficial effects of the mutations in AHR actually act through an effect on CYP1A1.

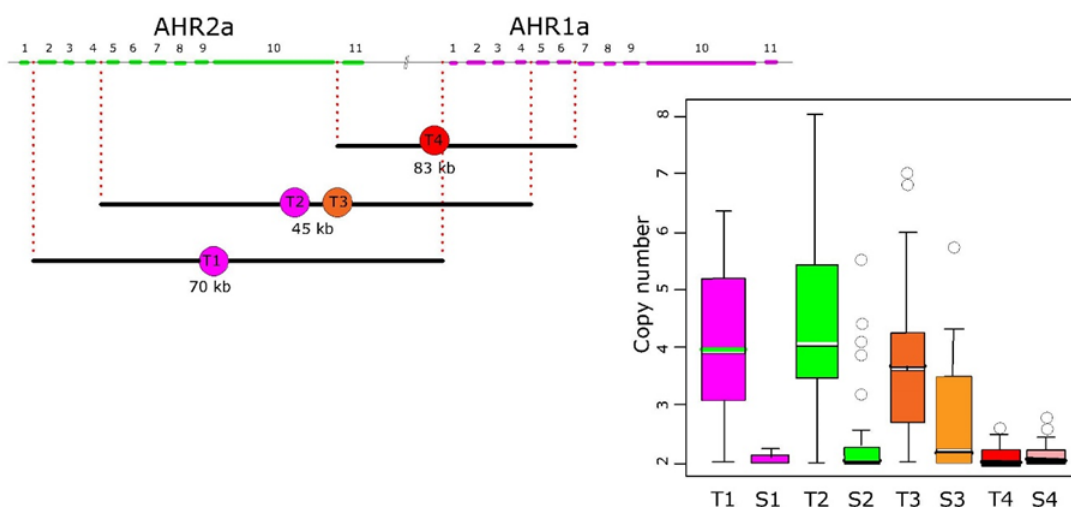


Figure 1. Showing the genetic variation among sensitive (S1 to S4) and tolerant (T1 to T4) populations of killifish, *Fundulus heteroclitus* along the East coast of the United States. Sensitivity and tolerance is towards sediments with high loads of PCBs and/or PAHs. The genome of *Fundulus* encodes four AHR (aryl hydrocarbon receptor) paralogs of which two are positioned in tandem, AHR2a and AHR1a, which carry long deletions (three different ones), indicated by black bars in the left figure. In addition, the populations have variable number of duplications of the CYP1A1 genes (right figure), not present to the same degree in the sensitive populations. Knock-out of AHR2a is protective of PCB and PAH toxicity, while duplication of CYP1A1 ensures a basal gene dose even when induction is less strong. Redrawn from Reid et al. (2016) by Wilma Ijzerman.

Interestingly, the various killifish populations show at least three different deletions in the AHR genes (Figure 1). In addition, the tolerant populations show various degrees of CYP1A1 duplication; in one population even eight paralogs are present. This can be interpreted as compensatory adaptations ensuring a basal constitutive level of CYP1A1 protein to conduct routine metabolic activities. The killifish example shows a wonderful case of interplay between genetic tinkering, and strong selection emanating from a polluted environment.

## Conclusion

In this module we have focused on genetic variation in the phase I enzyme, cytochrome P450. A similar complexity lies behind the phase II enzymes and the various xenobiotic-induced transporters (phase III). Still the P450 examples suffice to demonstrate that the machinery of xenobiotic metabolism shows a very large degree of genetic variation, as well as species differences due to duplications, deletions, gene conversion and lineage-specific selection. The variation resides both in copy number variation, alteration of coding sequences and in promoter or enhancer sequences affecting the expression of the enzymes. Such genetic variation is the template for evolution. In polluted environments enhanced expression is sometimes selected for (to neutralize toxic compounds), but sometimes also attenuated expression is selected (to avoid production of toxic intermediates). In the human

genome, many of the polymorphisms have a medical significance, determining a personal profile of drug metabolism and tendencies to develop cancer.

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### 4.2.14. Question 1

Comment upon: "In the human cytochrome P450 gene 1A1 133 single nucleotide polymorphisms have been described, of which 23 are non-synonymous".

- What is a "single nucleotide polymorphism" (SNP)?
- What is the difference between a synonymous SNP and a non-synonymous SNP?
- What could be the differences of nucleotide substitutions (1) in the promoter of CYP1A1, (2) in one of the introns of the CYP1A1 gene, (3) in one of the exons of CYP1A1?
- Does a single amino acid change in the cytochrome P450 protein have any medical relevance?

### 4.2.14. Question 2

Some populations of killifish along the Atlantic coast of the U.S. show very high resistance against organic contaminants such as dioxin-like PCBs. Genetic research has shown that these resistant populations have a deletion in the gene encoding aryl hydrocarbon receptor (Ahr). Explain why such a mutation can cause resistance to dioxin-like PCBs.

### 4.2.14. Question 3

Comment upon the concept of "Evolutionary rescue from pollution"- the evolution of tolerance as a way to diminish toxic effects of pollution.

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