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Reactive Metabolites: Their Nature and Toxicity

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1. Phase I and Phase II metabolism producing reactive intermediates
2. Bioactivation to radicals
3. Reactive oxygen species during inflammation
4. Conclusion

Bioactivation to reactive intermediates

- The metabolic formation of reactive intermediates is catalyzed by different enzymes; the majority involves cytochrome P-450 mediated oxidations.

- Reactive intermediates may react with low and high molecular weight cellular constituents. These interactions result in formation of less reactive chemicals and thus in detoxication or perturb critical cellular functions resulting in acute and/or chronic toxic effects.

- The toxicity and carcinogenicity of many chemicals is associated with the formation of reactants and covalent binding of reactive metabolites to DNA, lipids or proteins.

Reactive intermediates include chemically diverse functionalities such as

Epoxides

Quinones

Acyl halides

Carbocations

Nitrenium ions

α,β-unsaturated aldehydes

Reactive oxygen species

Bioactivation of xenobiotics comprises four categories, which describe basic types of reactive intermediates formed

Mechanism	**Structure and reactivity of the intermediate**	**Examples**
1. Biotransformation to **stable but toxic metabolites**	different structures, selective interaction of metabolites with specific acceptors or disruption of specific biochemical pathways	dichloromethane acetonitrile parathion
2. Biotransformation to **electrophiles**	reactive electrophiles	chloroform dimethylnitrosamine acetaminophen
3. Biotransformation to **free radicals**	radicals	carbon tetrachloride
4. Formation of **reactive oxygen species**	radicals	paraquat aromatic nitro-compounds inflammation

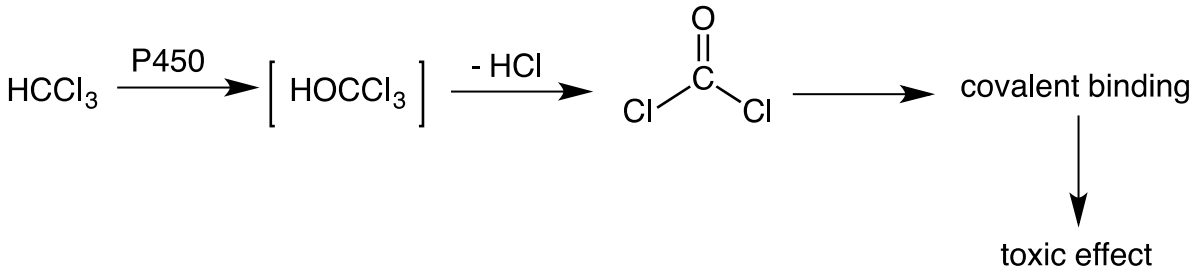
Phase-I-Enzymes

Oxidoreductases
Cytochromes P450 (CYP)
Flavin-containing monooxygenases (FMO)
Monoamine oxidases (MAO)
Cyclooxygenases (COX)
Dihydrodioldehydrogenases
DT-Diaphorases (NQOR)
Alcohol dehydrogenases (ADH)
Aldehyde dehydrogenases (ALDH)
NADPH P450 oxidoreductase (OR)
Hydrolases
Esterases
Amidases
Glucuronidases
Epoxidhydrolases (EH)

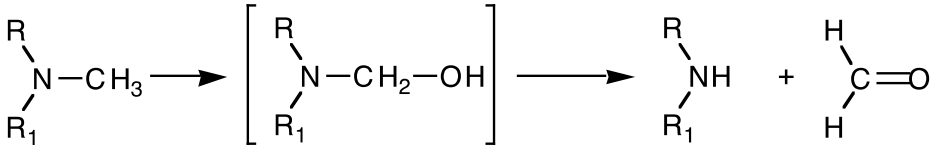
Phase-II Enzymes

Glutathiontransferases (GST)
UDP-Glucuronosyltransferases (UGT)
Sulfotransferases (SULT)
Acetyltransferases (NAT)
Methyltransferases
Aminoacyltransferases

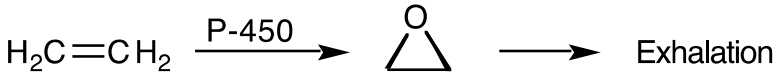
P450 dependent activation to reactants



Biotransformation of chloroform to phosgene

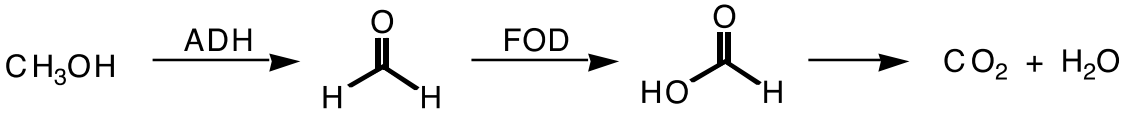


Oxidative dealkylation of alkylamines to formaldehyde

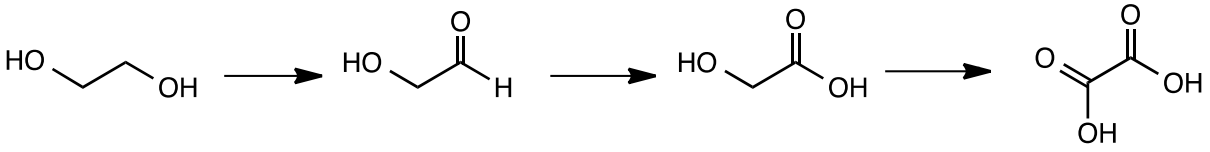


Oxidation of of ethylene to ethylene oxide

Alcohol- and Aldehyd-Dehydrogenases



Metabolism of methanol via formaldehyde to formic acid



Metabolism of 1,2-ethanediol by ADH to glyoxylic acid and oxalate

Bioactivation & Detoxication

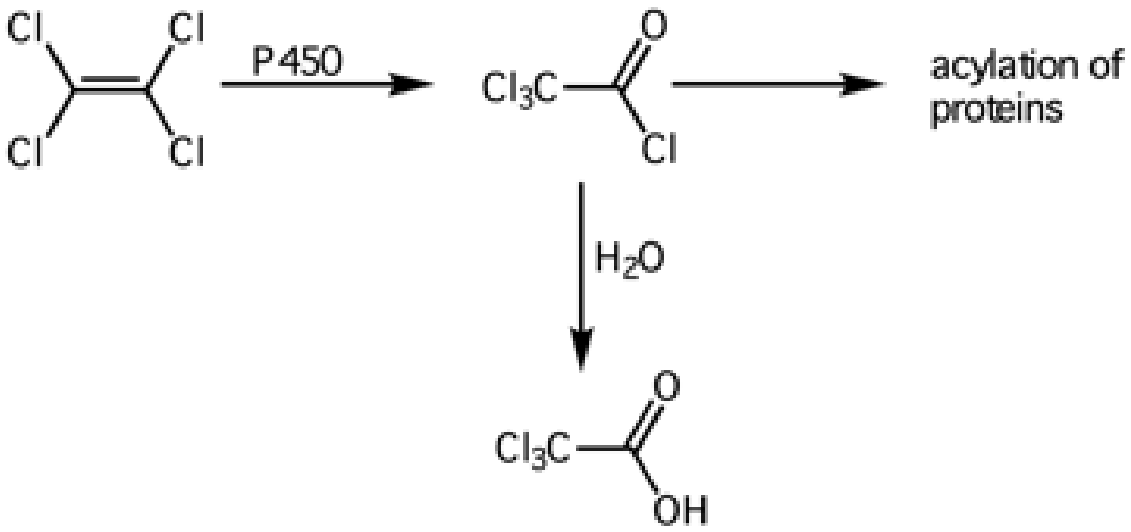
- Many chemicals, reactive metabolites formed during bioactivation are efficiently detoxified.

- Detoxication of reactive intermediates includes hydrolysis, glutathione conjugation or interactions with cellular antioxidants.

- Toxic effects occur when the balance between the production of reactive metabolites and their detoxification is disrupted: Saturation of inactivating enzymes, depletion of cofactors.

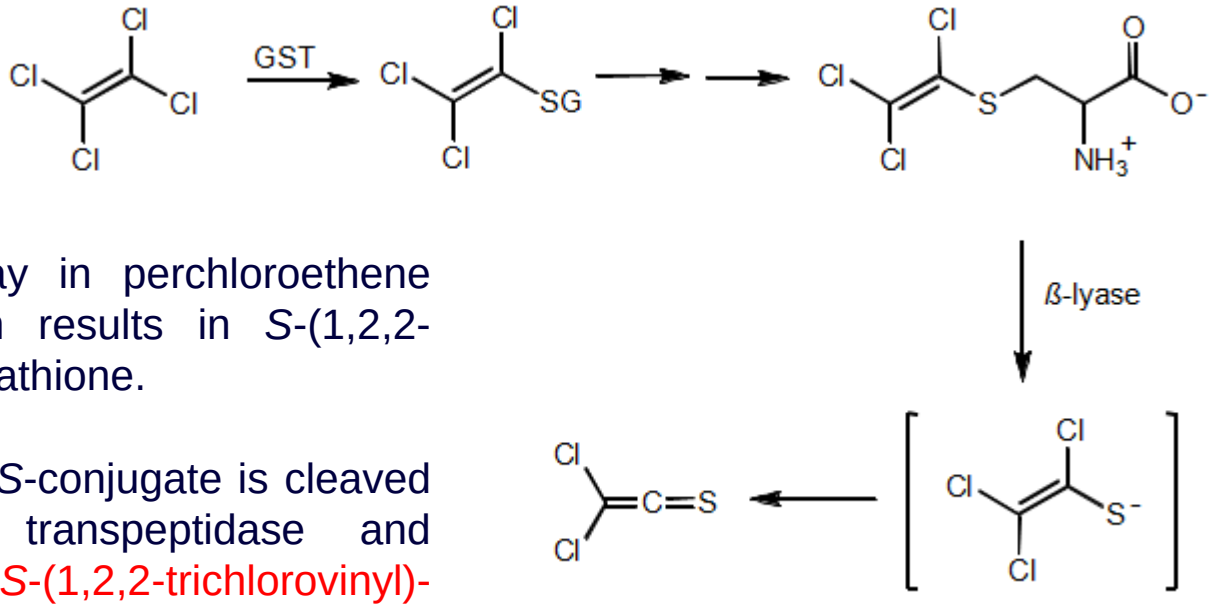
- However, conjugation may lead to reactive intermediates.

Detoxication of reactive intermediates due to hydrolysis



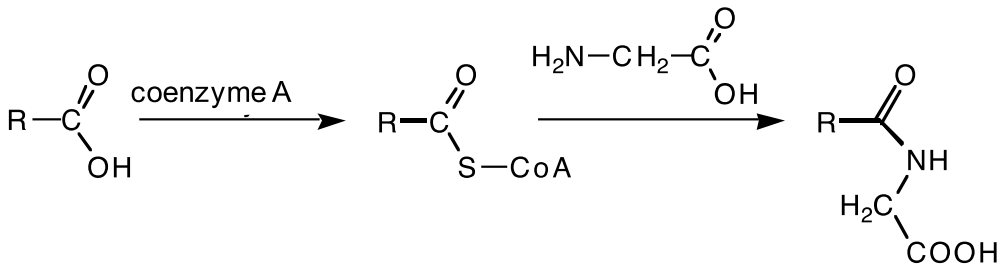
- The reaction of electrophilic xenobiotics with the nucleophile water is the simplest detoxication.
- Many of the products thus formed are of low reactivity and may be rapidly excreted.
- For example, acyl halides formed by the oxidation of olefins such as perchloroethene are hydrolyzed rapidly to halogenated carboxylic acids.
- Only minor amounts of the intermediary acyl halide reacts with protein and lipids

GSH dependent bioactivation

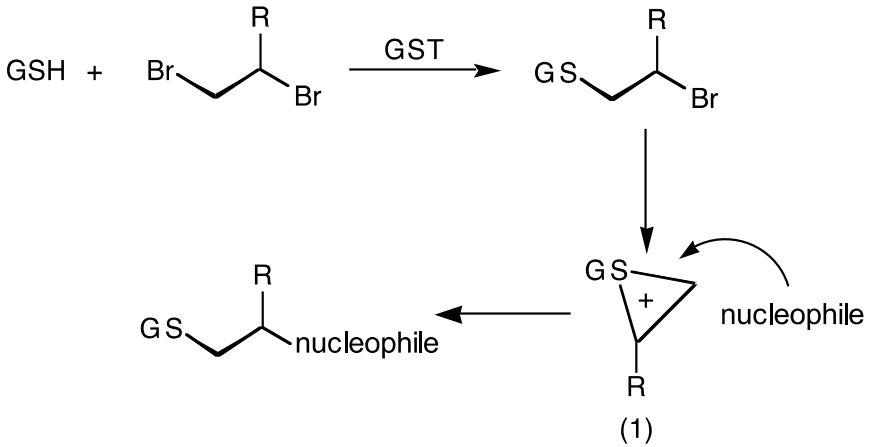


- A minor pathway in perchloroethene biotransformation results in *S*-(1,2,2-trichlorovinyl)glutathione.
- This glutathione *S*-conjugate is cleaved by g-glutamyl transpeptidase and dipeptidases to *S*-(1,2,2-trichlorovinyl)-L-cysteine, which is a substrate for renal ß-lyase .
- Cleavage by ß-lyase yields pyruvate, ammonia and a reactive thioketene which binds to cellular macromolecules explaining renal toxicity of perchloroethene.

Formation of reactants via conjugation

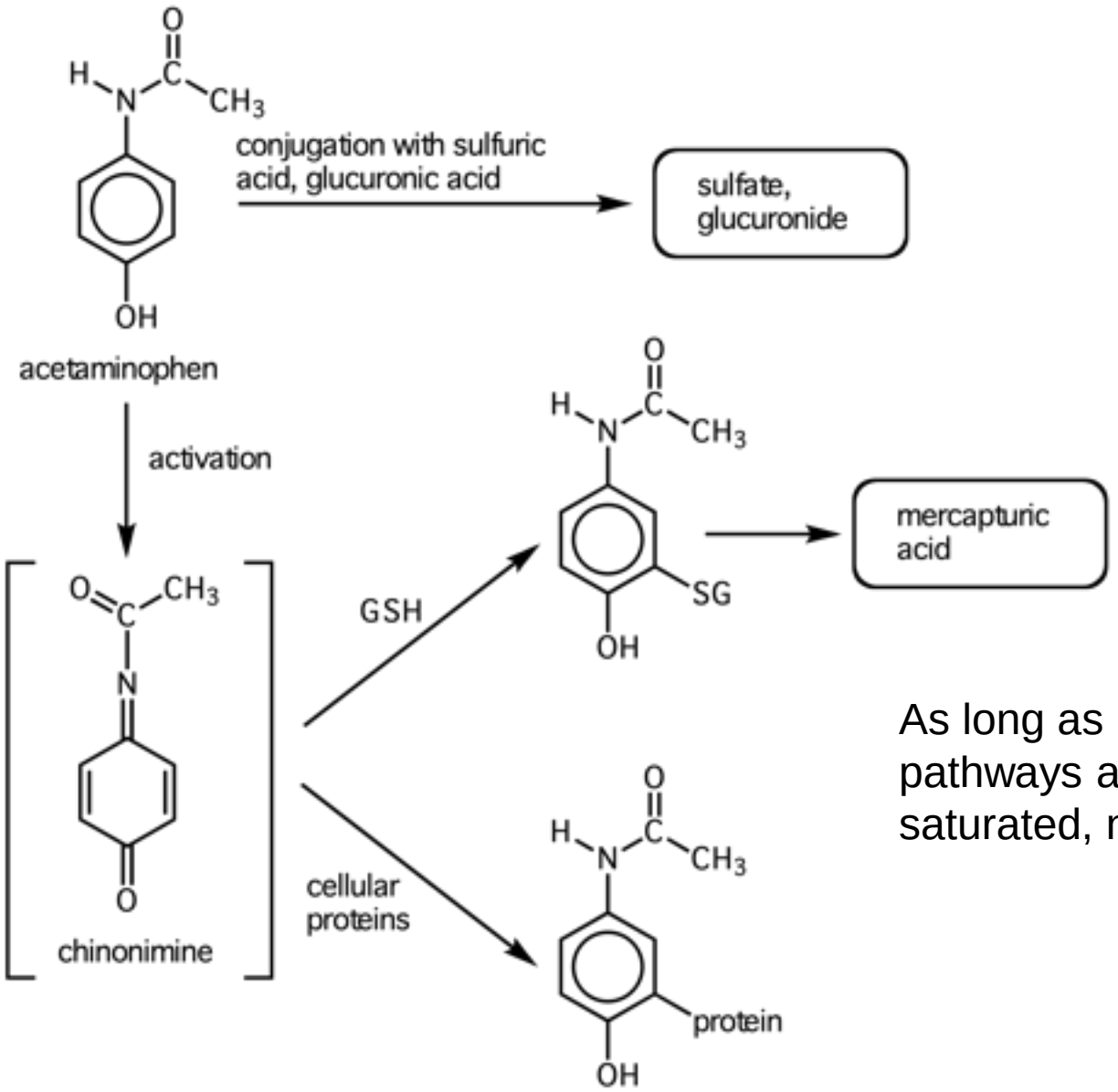


Activation of carboxylic acids forming amino acid conjugates



Activation of 1,2-dibromoalkanes by glutathione conjugation forming episulfonium ion (1) which reacts with nucleophilic macromolecules

Detoxication of reactive intermediates through GSH conjugation



As long as inactivation pathways are not saturated, no toxicity.

Bioactivation to radicals

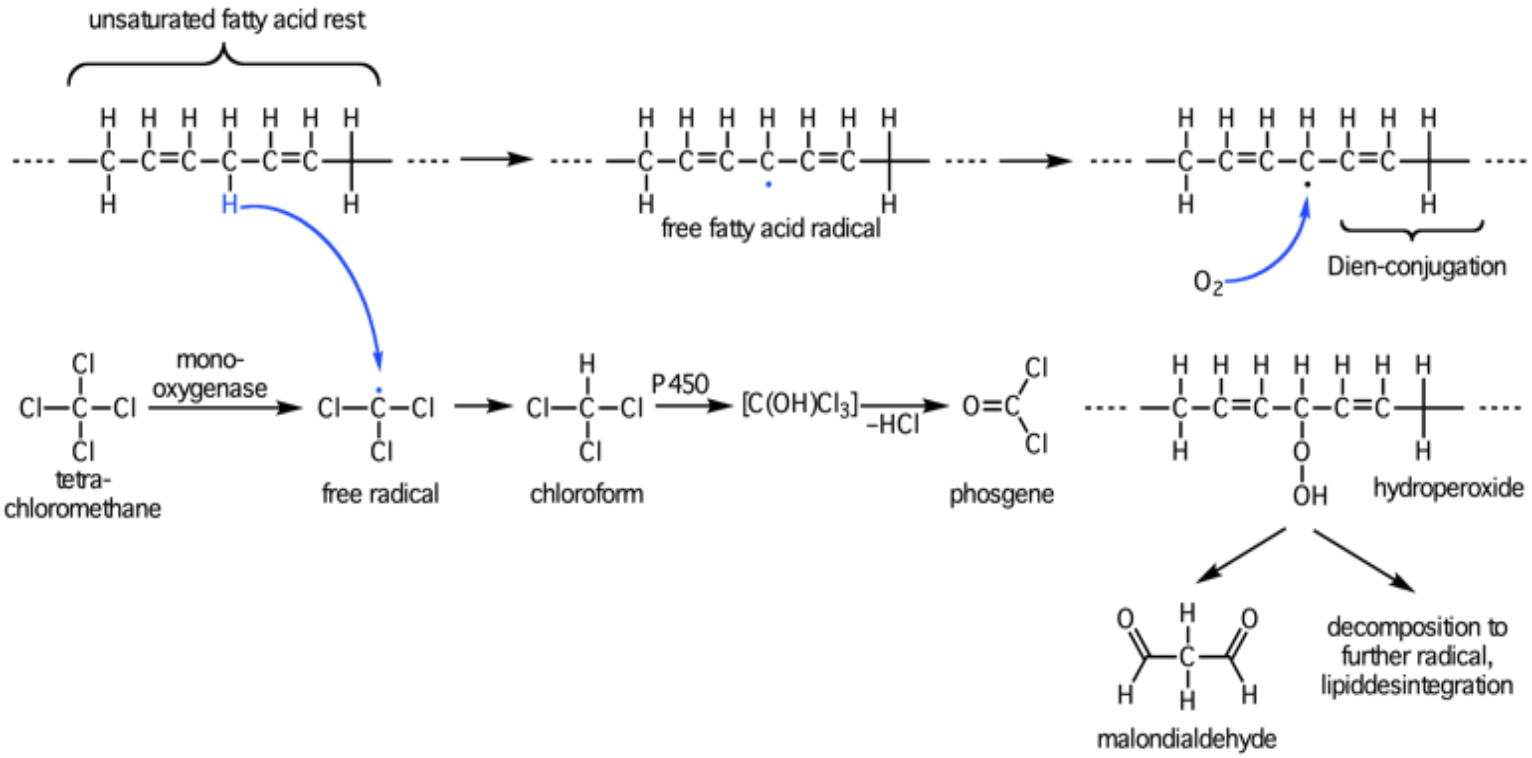
Radicals may be formed by the NADPH-dependent cytochrome P-450 reductase, by nitroreductases or by one-electron oxidations catalyzed by peroxidases such as prostaglandin synthetases.

Free radicals are highly reactive and, when formed in biological systems, react with a variety of tissue molecules.

Radicals may abstract hydrogen atoms, undergo oxidation-reduction reactions, dimerisation and disproportionation reactions.

Radicals may also participate in a chain mechanism, which is initiated by a reaction causing a free radical and propagated by a subsequence of reactions causing further radicals as products.

Carbon tetrachloride: bioactivation to a free radical and initiation of lipid peroxidation



Lipid peroxidation leads to inflammation and fibrosis after administration of carbon tetrachloride for 4wks.

Redox cycling and reactive oxygen metabolites

- Xenobiotics that undergo redox cycling or enzyme catalyzed oxidation-reduction reactions may lead to the production of reduced oxygen metabolites.

- Xenobiotic induced formation of reduced oxygen metabolites such as the
  - superoxide radical anion
  - hydrogen peroxide
  - hydroxyl radical

  may produce cell damage (oxidative stress). Consequences are:

* Decrease in GSH/GSSG ratio; * Lipid peroxidation;

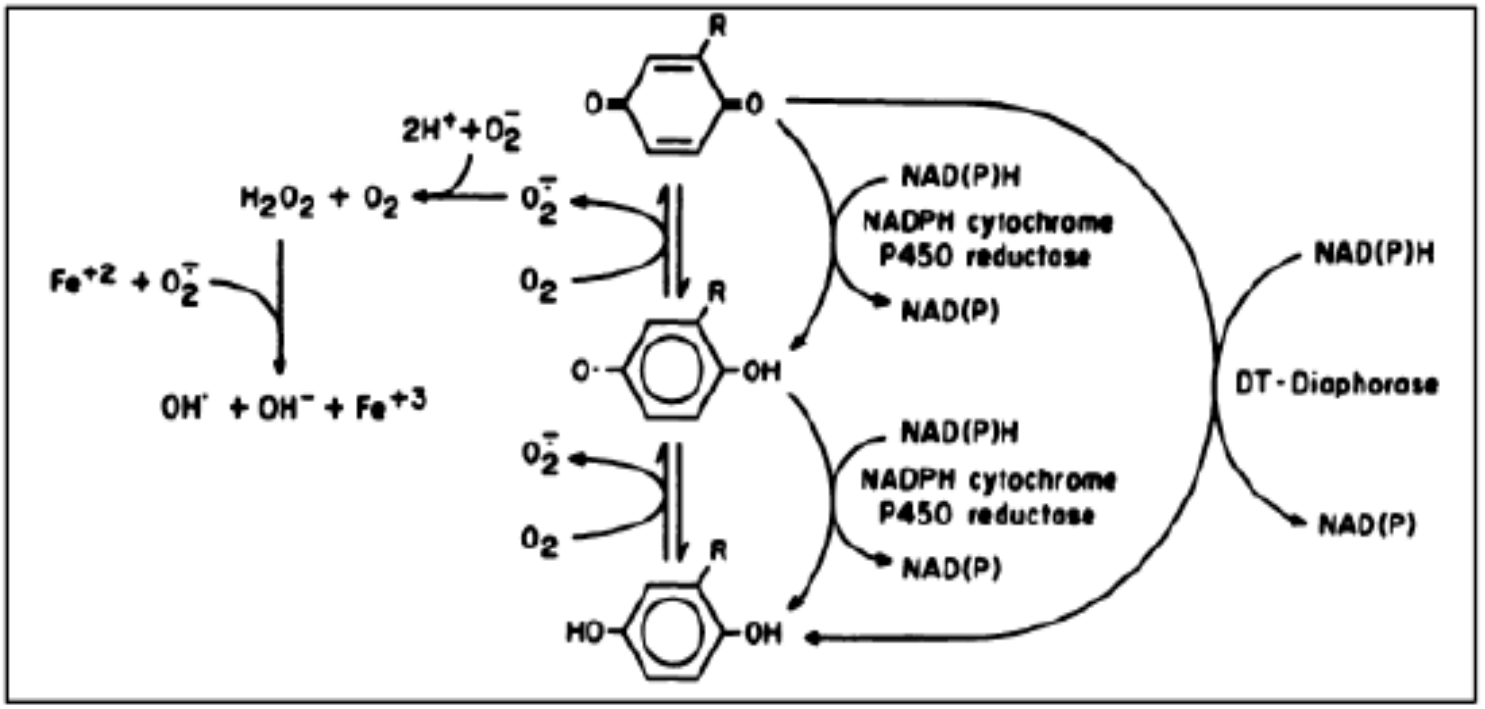
* Oxidation of protein thiols; * Inactivation of enzymes;
* Oxidative DNA damage; * Multiple other oxidations

Toxification of benzenetriol (1,2,4-THB) via redox cycling and concomitant production of reactive oxygen species.

1,2,4-THB can be oxidized by molecular oxygen to a semiquinone with the concomitant generation of superoxide radicals. In cellular systems the semiquinone is enzymatically reduced by NADPH-cytochrome P-450 reductase back to 1,2,4-THB or further oxidized to the quinone, again accomplished with superoxide formation.

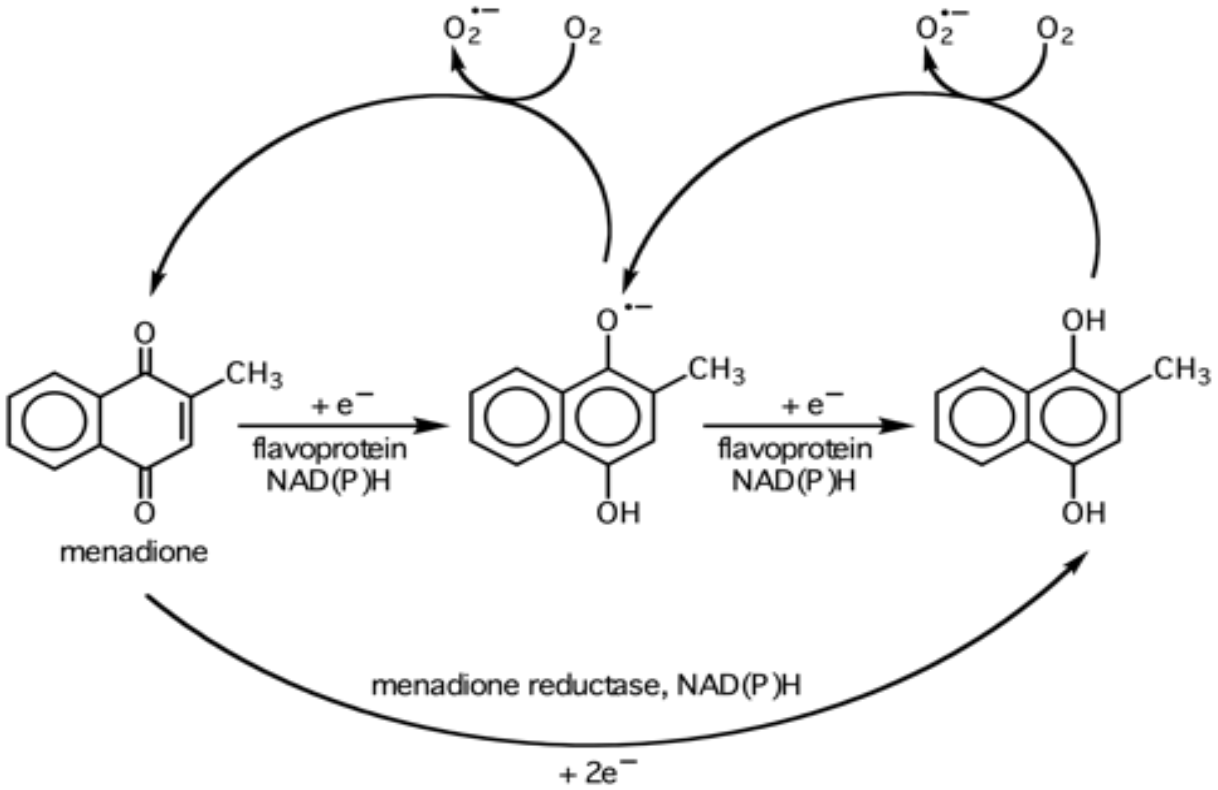
In the presence of transition metal ions superoxide radicals further react with hydrogen peroxide to yield hydroxyl radicals. (Pellack-Walker and Blumer 1986)

Benzoquinone: R = H; benzenetriol: R = OH

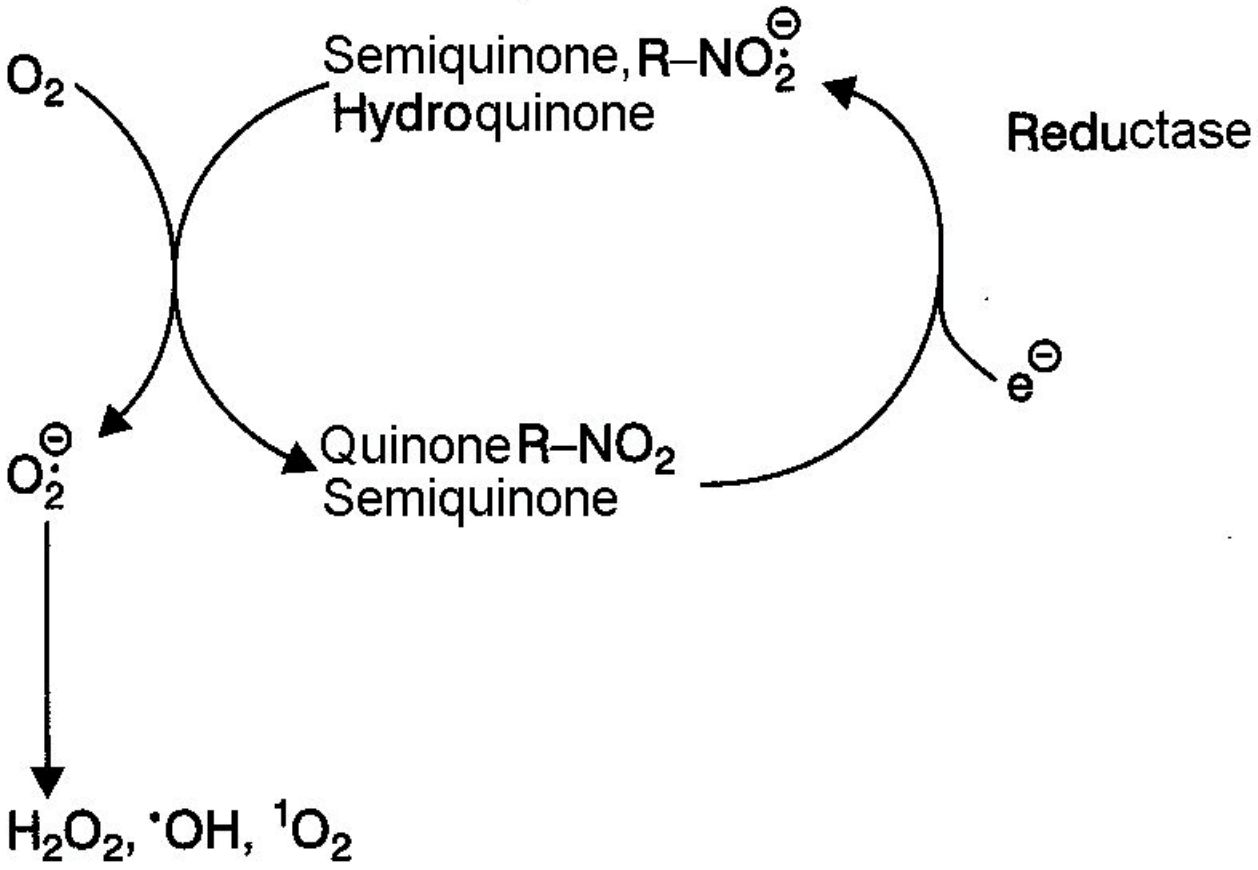


Formation of reactive oxygen metabolites through redox cycling of menadione

- 2-Methylnaphtoquinone (Menadione) and other quinones undergo enzymatic redox cycling
- These one-electron oxidation reactions are associated with the formation of the superoxide radical anion ($O_2^{-\cdot}$) by one electron reduction of triplet oxygen



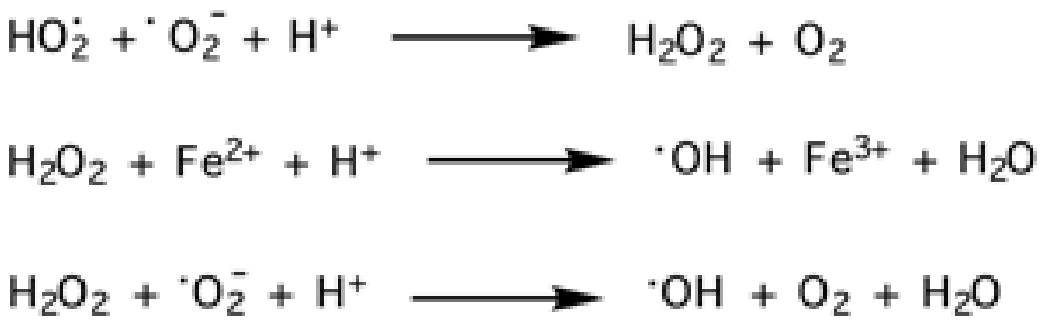
Formation of superoxide radical and further oxygen species by redoxcycling of an aromatic nitro compound.



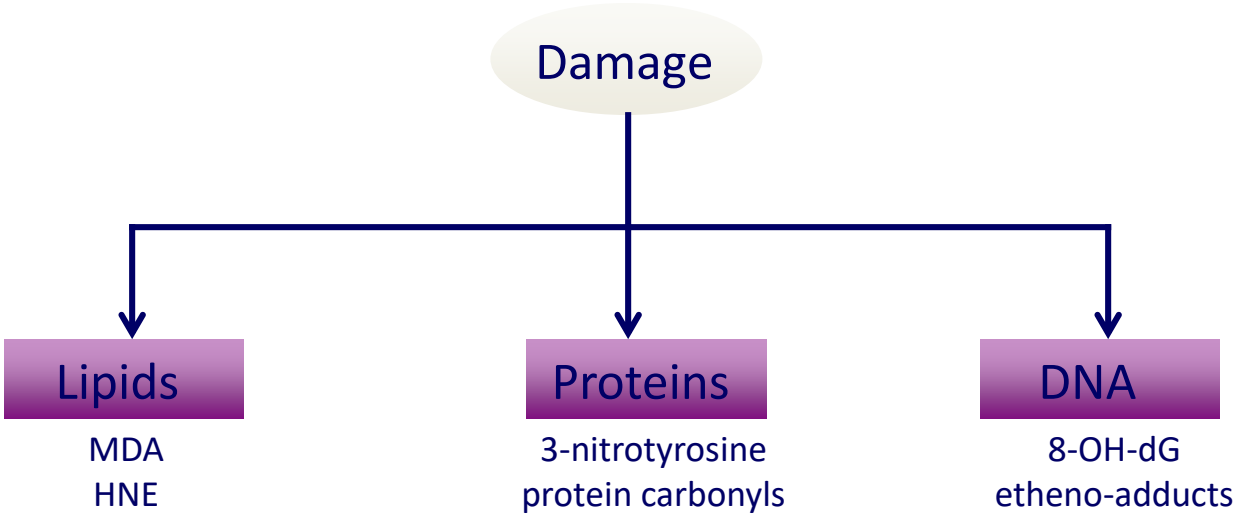
Reduction by one electron transitions and reaction with oxygen leads to formation of a superoxide radical, subsequently to H_2O_2, hydroxyl radicals and singlet oxygen.

Reactivity of oxygen metabolites

- In aqueous solution, superoxide is not impressively reactive
- Further reduction of superoxide may result in hydrogen peroxide
- Hydrogen peroxide is also a poor oxidant in biological systems, but sufficiently stable to cross biological membranes.
- Hydrogen peroxide can give rise to the hydroxyl radical by the Fenton-reaction catalyzed by metal ions such as Fe^{2+} (M = transition metal).
- The highly reactive hydroxyl radical may then initiate cellular damage by radical based mechanisms.



Toxicity mediated by reactive oxygen species (ROS)



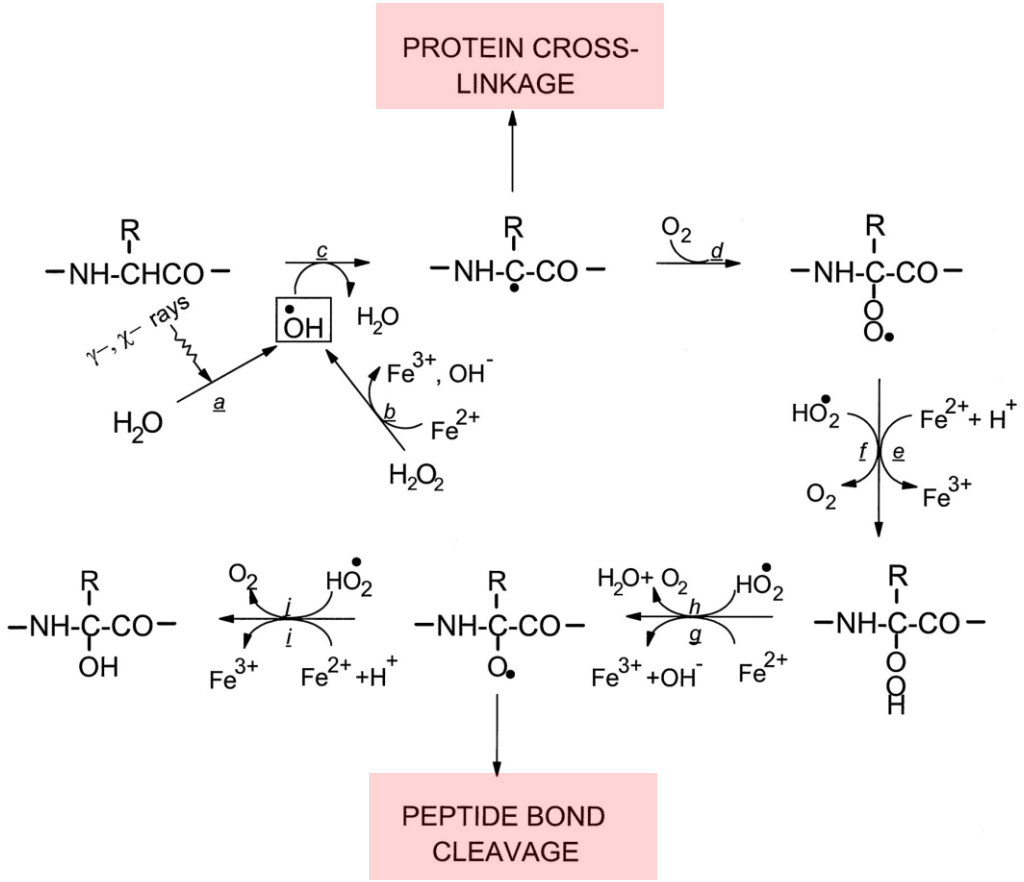
ROS and oxidative damage of proteins

- **Oxidation of the amino acid side chain**

Amino acids	Oxidation products
Cysteine	Disulfides, cysteic acid
Methionine	Methionine sulfoxide, methionine sulfone
Tryptophan	2-, 4-, 5-, 6-, and 7-Hydroxytryptophan, nitrotryptophan
Phenylalanine	2,3-Dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine
Tyrosine	3,4-Dihydroxyphenylalanine, tyrosine-tyrosine cross-linkages, Tyr-O-Tyr, cross-linked nitrotyrosine
Histidine	2-Oxohistidine, asparagine, aspartic acid
Arginine	Glutamic semialdehyde
Lysine	α-Aminoadipic semialdehyde
Proline	2-Pyrrolidone, 4- and 5-hydroxyproline pyroglutamic acid,
Threonine	2-Amino-3-ketobutyric acid
Glutamyl	Oxalic acid, pyruvic acid

Oxidative damage of proteins

Oxidation of the Protein Backbone is initiated by the *OH-dependent abstraction of the α-hydrogen atom of an amino acid residue to form a carbon-centred radical or oxygen radical.



Reactive oxygen species in inflammation and tissue injury

Mittal et al 2014, Antioxid Redox Signal 20, 1126-67

Reactive oxygen species (ROS) play an important role in the progression of inflammatory disorders.

ROS are generated by polymorphonuclear neutrophils (PMNs) at the site of inflammation causing endothelial dysfunction and tissue injury. Under the inflammatory conditions, oxidative stress produced by PMNs leads to the opening of inter-endothelial junctions and promotes the migration of inflammatory cells across the endothelial barrier. The migrated inflammatory cells help in the clearance of pathogens and foreign particles but also lead to tissue injury.

Formation of reactive oxygen species during inflammation

Production of reactive oxygen species (ROS) is central to the progression of many inflammatory diseases. The ROS produced by cells such as polymorphonuclear neutrophils (PMNs) promote endothelial dysfunction by oxidation of crucial cellular signaling proteins such as tyrosine phosphatases.

The biologically relevant ROS are

- superoxide anion ($O_2^{\bullet -}$)
- hydroxyl radical ($OH^{\bullet}$)
- hydrogen peroxide (H_2O_2)
- hypochlorous acid (HOCl)

Functions of Hydroxyl Radicals

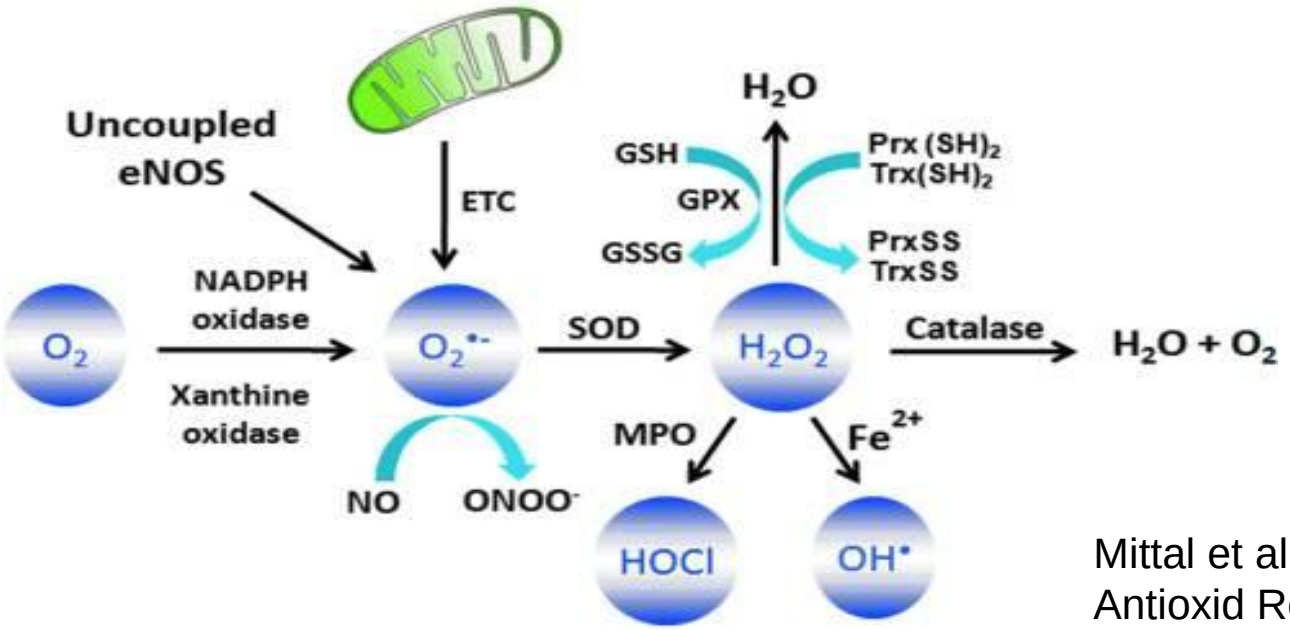
Hydroxyl radicals can induce a variety of oxidative reactions.

Balance their unpaired electron configuration through directly adding themselves (in the form of OH group) for example to **unsaturated or double, triple bonds, such as C=C, C=O, S=O, N=N.**

Hydroxylation (introduction of a hydroxyl group - OH- into various organic compounds).

Formation of a various types of secondary radicals, according to the reaction: $\mathbf{OH\cdot + RH \rightarrow H_2O + R\cdot}$.

In practice, the multitude of the variations of such possible reactions in almost limitless.



Mittal et al 2014:
Antioxid Redox Signal
20, 1126-1167

Haber-Weiss reaction

$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$ (step 1)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$ (step 2, Fenton's reaction)

Antioxidant reactions:

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ *(Superoxide dismutase)*

$2\,H_2O_2 \rightarrow H_2O + O_2$ *(Catalase)*

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ *(Glutathione peroxidase)*

$H_2O_2 + Prx\,(SH)_2 \rightarrow 2H_2O + PrxSS$ *(Peroxidredoxin)*

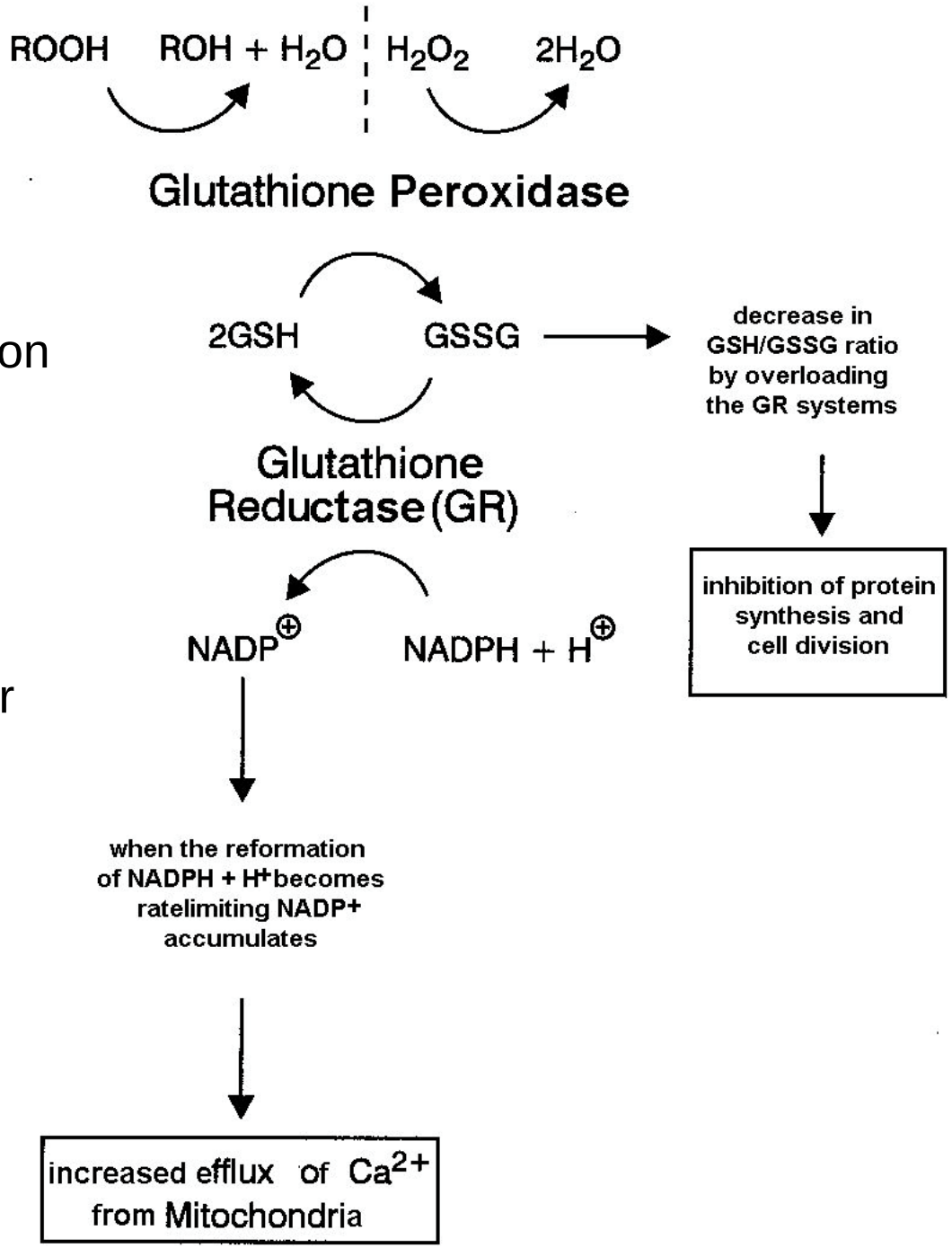
$Prx\,SS + Trx(SH)_2 \rightarrow Prx\,(SH)_2 + TrxSS$ *(Thioredoxin)*

Superoxide is produced by NADPH oxidase/xanthine oxidase-derived reduction of molecular oxygen, uncoupled endothelial nitric oxide synthase (eNOS), or mitochondrial electron transport chain (ETC).

$O_2^{\bullet-}$: superoxide anion; $OH^{\bullet}$: hydroxyl radical; H_2O_2 : hydrogen peroxide;
HOCl: hypochlorous acid

Inactivation of H_2O_2

GSSG generated during the glutathione peroxidase reaction is reduced to GSH by glutathione reductase at the expense of $NADPH_2$, and formation of $NADP^+$. When $NADPH_2$ formation becomes rate limiting, cellular concentrations of $NADP^+$ and GSSG increase resulting in oxidative stress.



Conclusion I

In biological systems an array of reactive intermediates are formed that may interact with cellular components as well as chemicals.

These result from Phase I and Phase II metabolizing enzymes and the formation of reactive oxygen species formed during physiological and pathophysiological processes such as redox cycling or inflammation.

Many reactants are inactivated, either by reaction with inert macromolecules or via enzymes.

Conclusion II

The scavenging systems are effective as long as as co-factors such as GSH are available.

At high concentrations such systems become overwhelmed and the inactivated free reactants induce toxicity, in case of reactive oxygen species via oxidative stress.

Understanding the balance between activation and inactivation requires information on the production rate of the reactant and the capacity of its scavenging system (enzyme kinetics).

This allows identification of a threshold at which the scavenging systems are not overwhelmed.

Acknowledgement

Several slides and formulas have been provided by Drs. Wolfgang Dekant and Angela Mally, University of Würzburg

Others are taken from *Toxicology and Risk Assessment* (Wiley 2018), chapters:

- Biotransformation (Wolfgang Dekant, Würzburg)
- Cytotoxicity (Daniel Dietrich, Konstanz)

Thank you

Publications on Skin Metabolism

Hu et al. 2010: Xenobiotic metabolism gene expression in the EpiDermin vitro 3D human epidermis model compared to human skin. Toxicol. In Vitro 24:1450–1463.

Bonifas et al 2010: Evaluation of cytochrome P450 1 (CYP1) and N-acetyltransferase 1 (NAT1) activities for the development of in vitro techniques for predictive testing of contact sensitizers. Toxicol in Vitro 24, 973-980

Oesch et al 2014: Xenobiotoc-metabolizing enzymes in the skin of rat, mouss, pig, guinea pig, man, and in human skin models. Arch Toxicol 88, 2135-2190

Marie et al 2019: Evaluation of risk modification for p-phenylenediamine sensitization by N-acetyltransferase 1 and 2 for two highly sensitive cases, *Contact Dermatitis*, 81, 138-140

Enzymes involved in activation of pro-sensitizers
(Blömeke et al., 2008)

Four enzyme classes that are described for pro-sensitizer activation, i.e.,

- Microsomal cytochrome P450 mono-oxygenases (CYPs),
- Alcohol dehydrogenases (ADH),
- Carboxyl-esterases (CE),
- Flavin-dependent mono-oxygenases (FMO).

Conjugating enzymes (such as N-acetyltransferases) catalyze detoxification

Bioactivation to radicals

Free radicals are chemical species which may be formed by

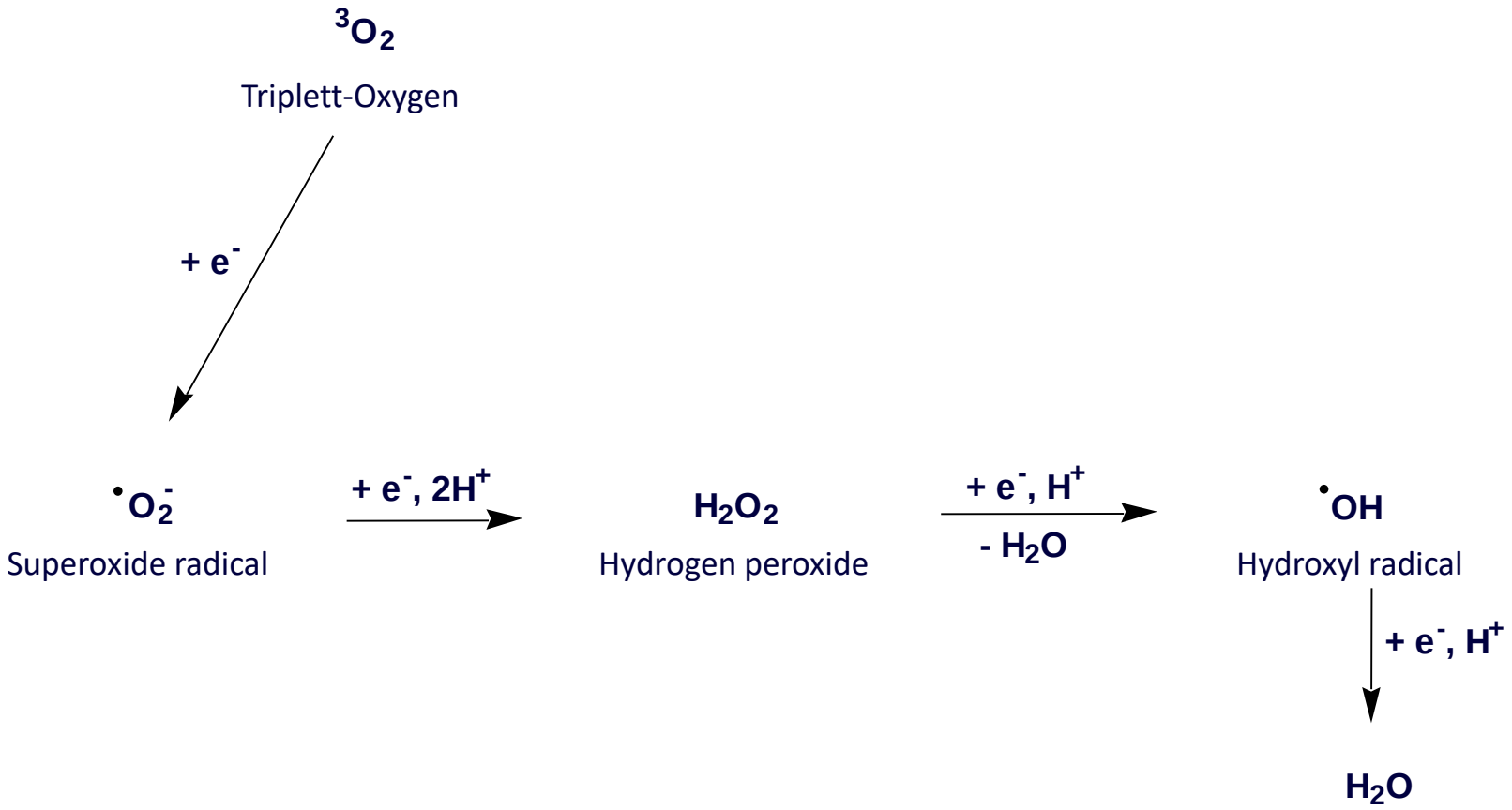
- a one-electron oxidation to yield a cation radical:

- by a one electron reduction to yield an anion radical:

- by homolytic fission of a s-bond to give a neutral radical:

Formation of reactive oxygen species

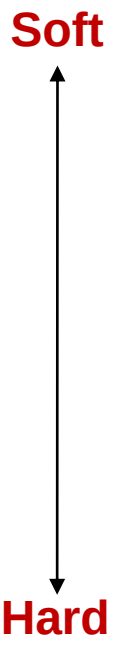
Reactive oxygen species formed by sequential one-electron reduction of molecular triplet-oxygen



Reactive electrophiles and their cellular targets

- Soft electrophiles (low charge density, highly polarizable) preferentially react with soft nucleophiles
- Hard electrophiles (high charge density, low polarizability) preferentially react with hard nucleophiles

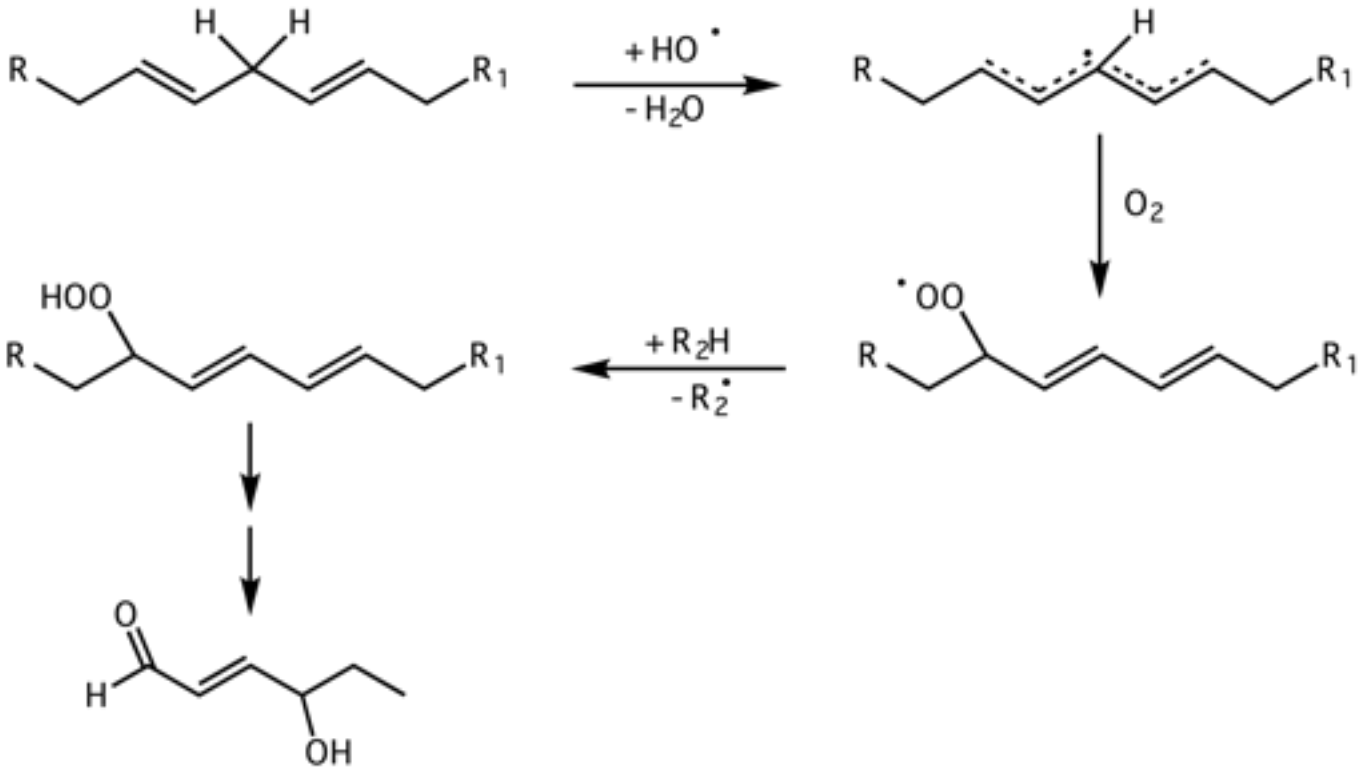
ELECTROPHILES	NUCLEOPHILES
Carbon in polarized double bonds (e.g. quinones, α,β-unsaturated aldehydes)	Sulfur in thiols (e.g. cysteinyl residues in proteins and glutathione)
Carbon in epoxides, aryl halides	Sulfur in methionine
Aryl carbonium ions	Nitrogen in primary and secondary amino groups of proteins
Benzylic carbonium ions, nitrenium ions	Nitrogen in amino groups in purine bases in nucleic acids
Alkyl carbonium ions	Oxygen of purines and pyrimidines in nucleic acids Phosphate oxygen in nucleic acids



Bioactivation to radicals

Chemical	Radical	Toxicity
Carbon tetrachloride	$\cdot CCl_3$	Hepatotoxicity
Paraquat	Paraquat radical cation + $O_2^{\cdot -}$	Pulmonary Toxicity
Daunomycin	Daunomycin radical + $O_2^{\cdot -}$	Cardiac Toxicity
Nitrofurantoine	$RNO_2^{\cdot -}$, $O_2^{\cdot -}$	Pulmonary Toxicity
3-Methylindole	3-Methylindole radical (N·)	Pulmonary Toxicity

ROS and lipid peroxidation



4-hydroxy-2-hexenal
(and other toxic aldehydes, e.g. malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE)

Test Systems for Skin Metabolism

Hu et al. 2010. Xenobiotic metabolism gene expression in the EpiDermin vitro 3D human epidermis model compared to human skin. Toxicol. In Vitro 24:1450–1463.

- Functional protein (enzyme) activity rarely proven.
- Dermal substrate turnover is assessed in vitro by distinct incubation protocols using different model substrates.

Ongoing discussion on the most accurate procedure:

- systemic incubation of the substrate in the culture media,
- topic application on skin models,
- incubation in vitro using decornified skin biopsies,
- subcellular fractions such as S9 and microsomes.