Centrum pro výzkum toxických látek v prostředí

BIOMARKERS AND TOXICITY MECHANISMS

12 – BIOMARKERS of EFFECTS

Luděk Bláha, PřF MU, RECETOX
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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
OP Vzdělávání pro konkurenceschopnost

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

In vivo biomarkers of effects / response

- Behavioral and Clinical biomarkers
- Pathology
- Clinical chemistry and hematology
- Enzymatic changes
- Gene and protein expression biomarkers
- Detoxification and oxidative stress markers

Behavioral and clinical biomarkers

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Examples of behavioral biomarkers

Table 7.4 Effect of some agricultural chemicals on behavioural parameters of the rainbow trout

Chemical	LD_{50} (96hr)	Swimming capacity	Swimming activity	Strike frequency	Daphnia consumed	% consuming daphnia	% survival from predation
Carbaryl	1.95	0.1–1	0.1–1	>1	0.1–1	0.1–1	<0.01
Chlordane	0.042	>0.02	0.002–0.02	0.002–0.02	0.002–0.02	0.002–0.02	0.002–0.02
DEF	0.66	0.05–0.1	0.005–0.05	0.005–0.05	<0.005	0.005–0.05	0.005–0.05
2,4-DMA	100	5–50	5–50	5–50	5–50	0.5–5	5–50
Methyl parathion	3.7	>0.1	<0.01	0.01–0.1	<0.1	0.01–0.1	0.01–0.1
Pentachlorophenol	0.052	>0.02	0.002–0.02	0.002–0.02	0.0002–0.002	>0.02	0.002–0.02

DEF: tributyl phosphorotrithioate
2,4-DMA: 2,4-dichlorophenoxyacetic acid
After Little *et al.* (1990).

Concentrations affecting behaviour: often lower than LD50

→ **early markers of (lethal) toxicity**

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Behavioral and clinical "biomarkers"

Interpretation

: are these really biomarkers ?
(**effects already demonstrated *in vivo?***)
= biomarkers of existing serious stress / intoxication

Parameters evaluated

- body weight
- food consumption
- fitness & welness

(Histo)pathology biomarkers

Pathology

(-) Destructive methods, Time consuming, Professional requirements

(+) High relevance – organ/tissue changes

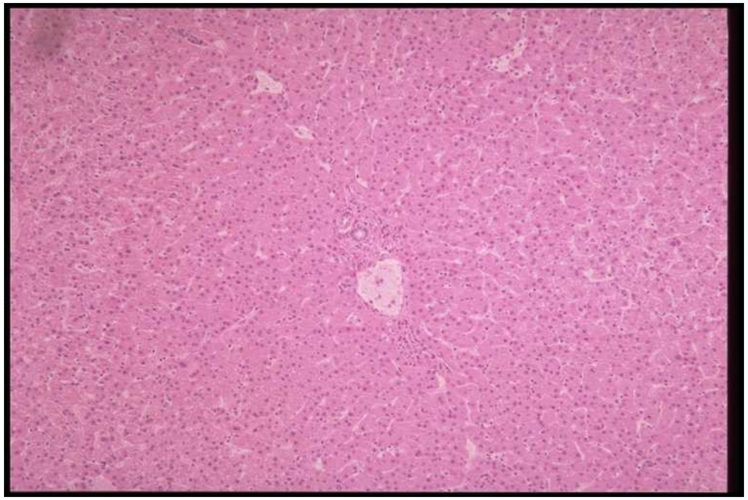
1) microscopy of internal organs

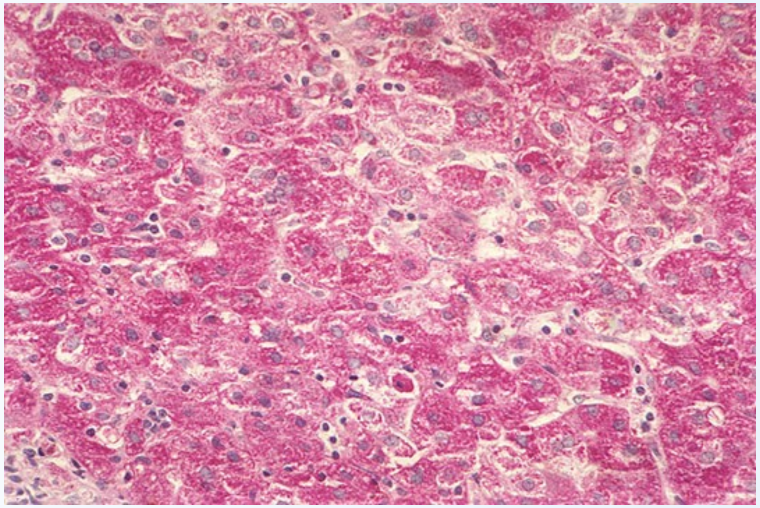
A) observations of **non-specific changes** in internal organs

B) specific **changes**, e.g.

in liver (dioxin-like POPs, cyanobacterial toxins ..)

intersex / imposex formation (xenoestrogenicity)





Example: Liver damage by cyanobacterial toxins microcystins

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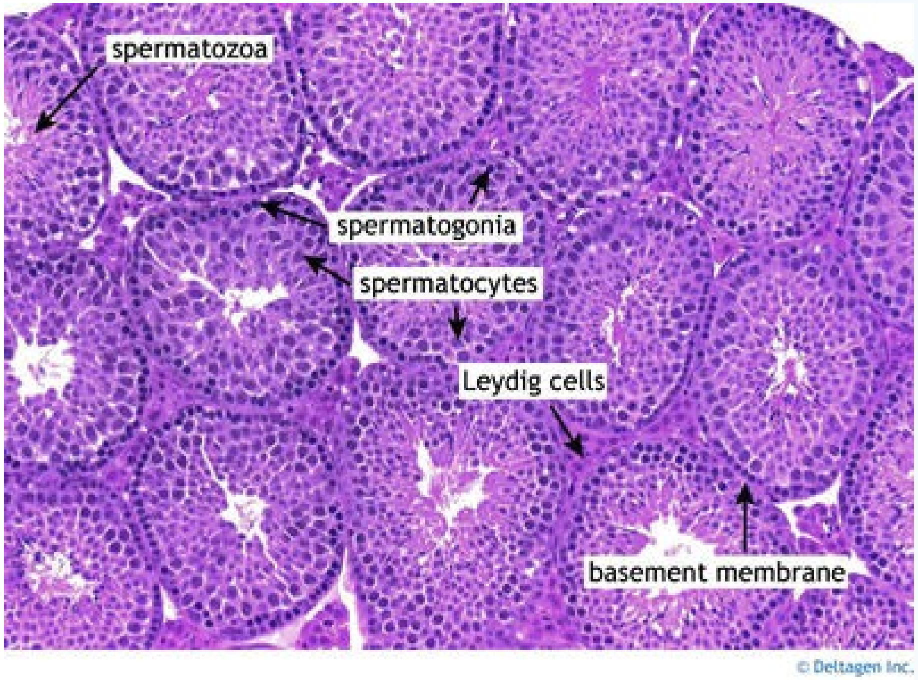
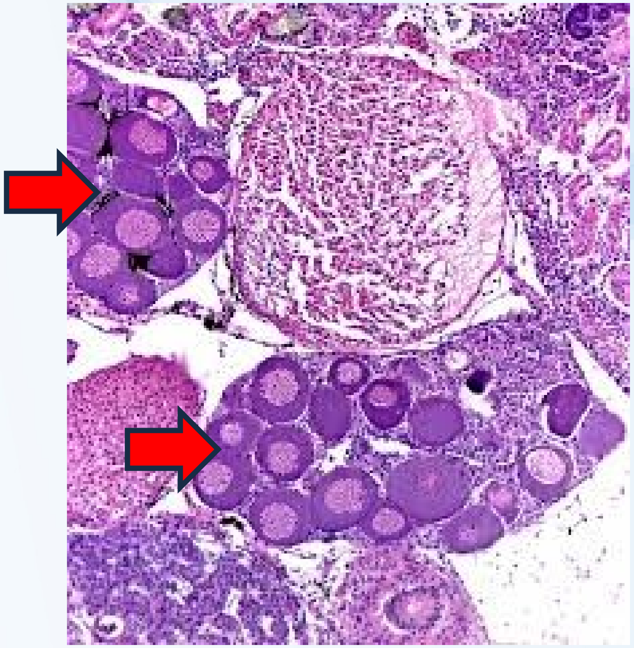
Endocrine disruption: Intersex microscopy

Testicular tissue



Oocytes within testis



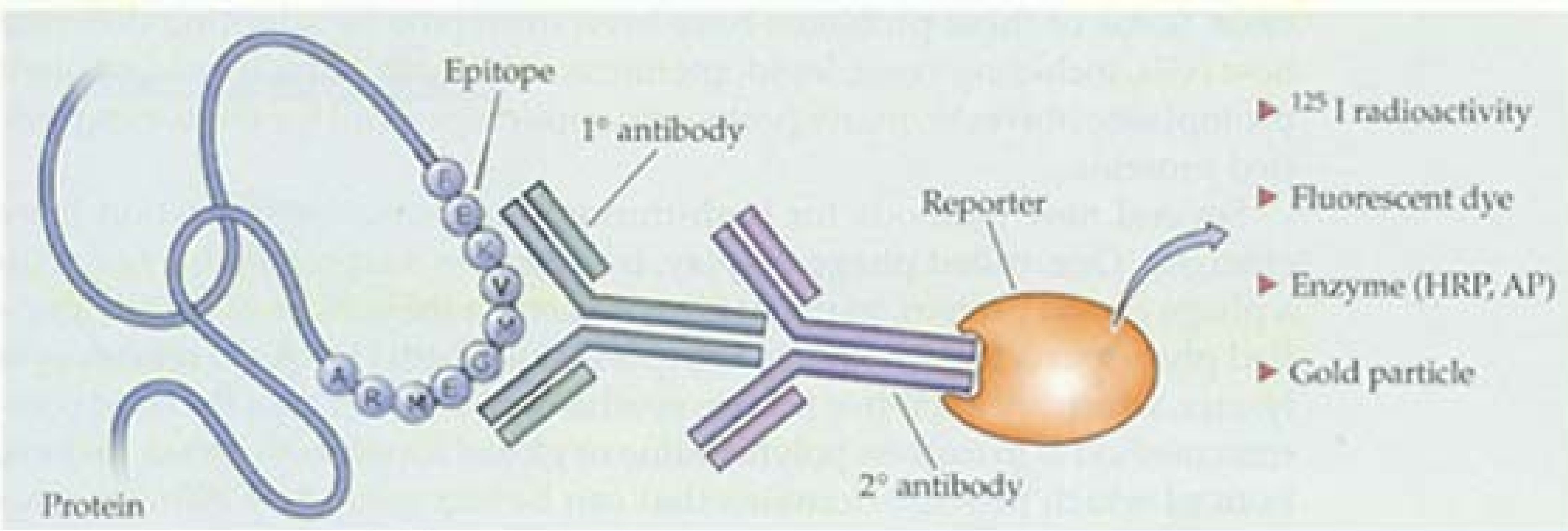
2) immunohistochemistry & microscopy

: determination of “specific” changes in tissues

: Fluorescein (FITC) - labeled antibodies (Ab) applications

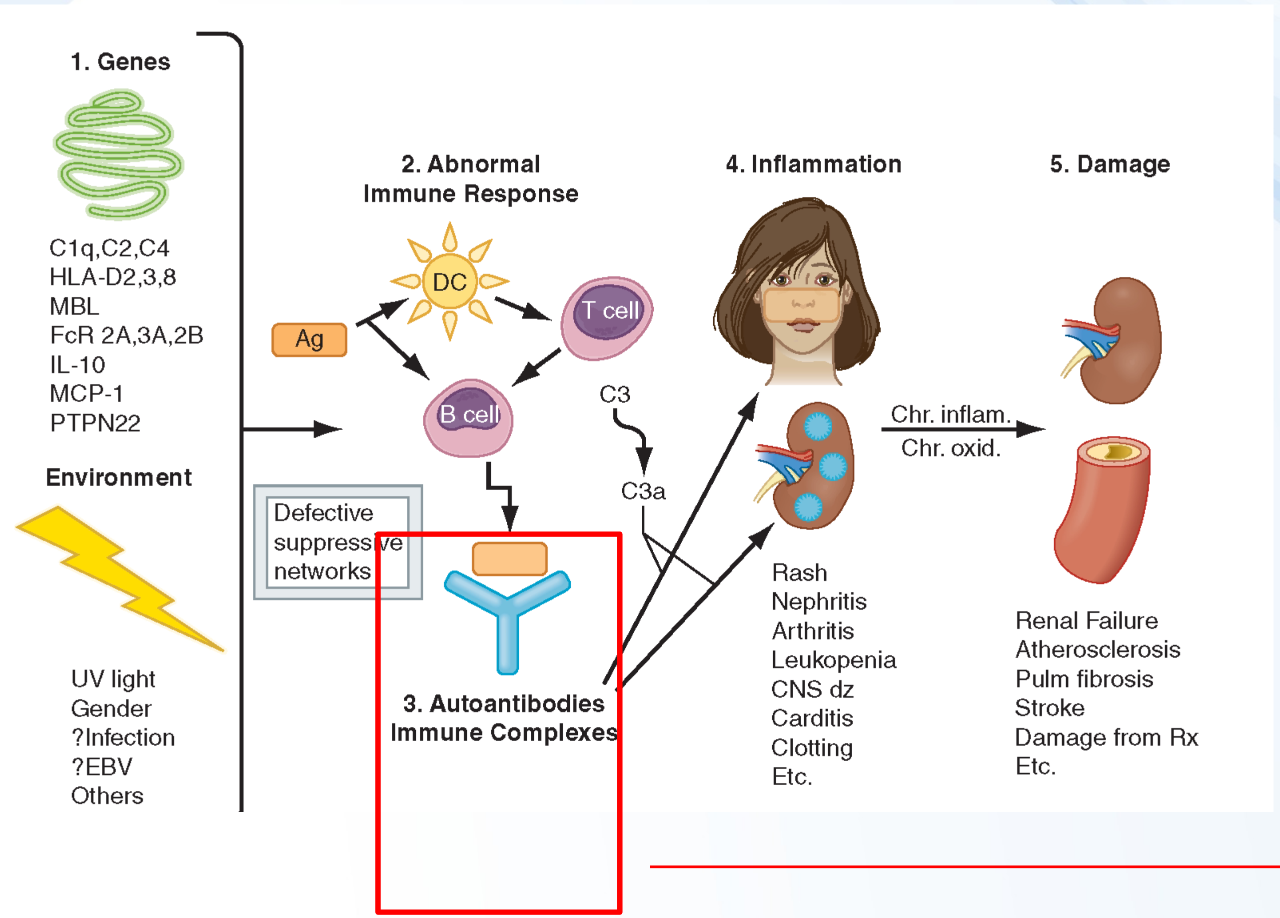
Example → toxicant induced autoimmunity:

anti-nuclear Ab (ANA test)



2) immunohistochemistry & microscopy
anti-nuclear Ab (ANA test)

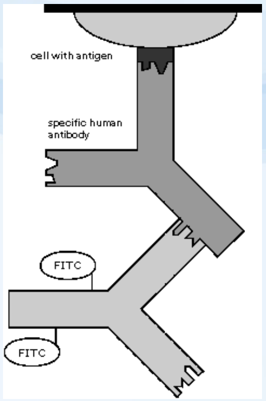
Systemic lupus

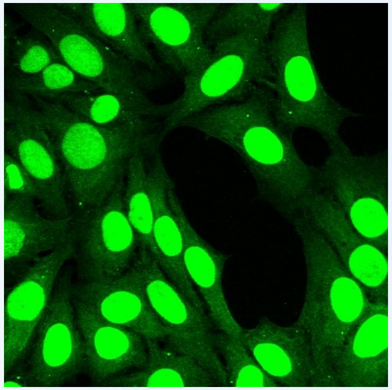


ANA test

* Determination of antibodies in patient blood acting against "nuclei" proteins (ANA)

: target: permeated liver cells on slide
: application of blood (Ab)
: visualization (secondary Ab)





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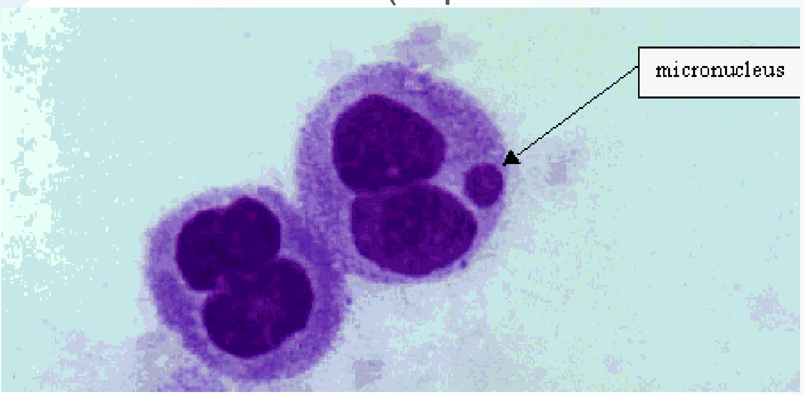
3) Nuclear DNA damage characterization

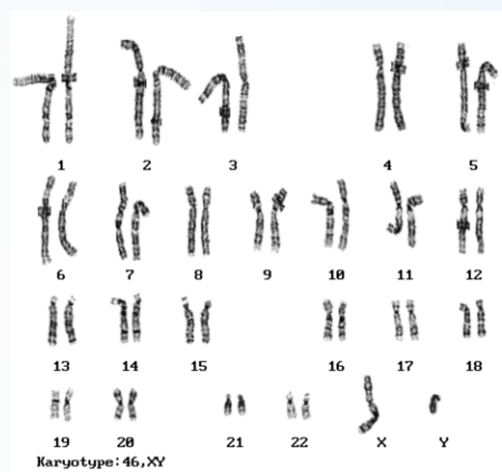
3.1. **micronuclei (MN) evaluation by microscopy**

: **example:** MNs in blood lymphocytes of hospital workers
(exposed to anticancer drugs – they are often carcinogenic)



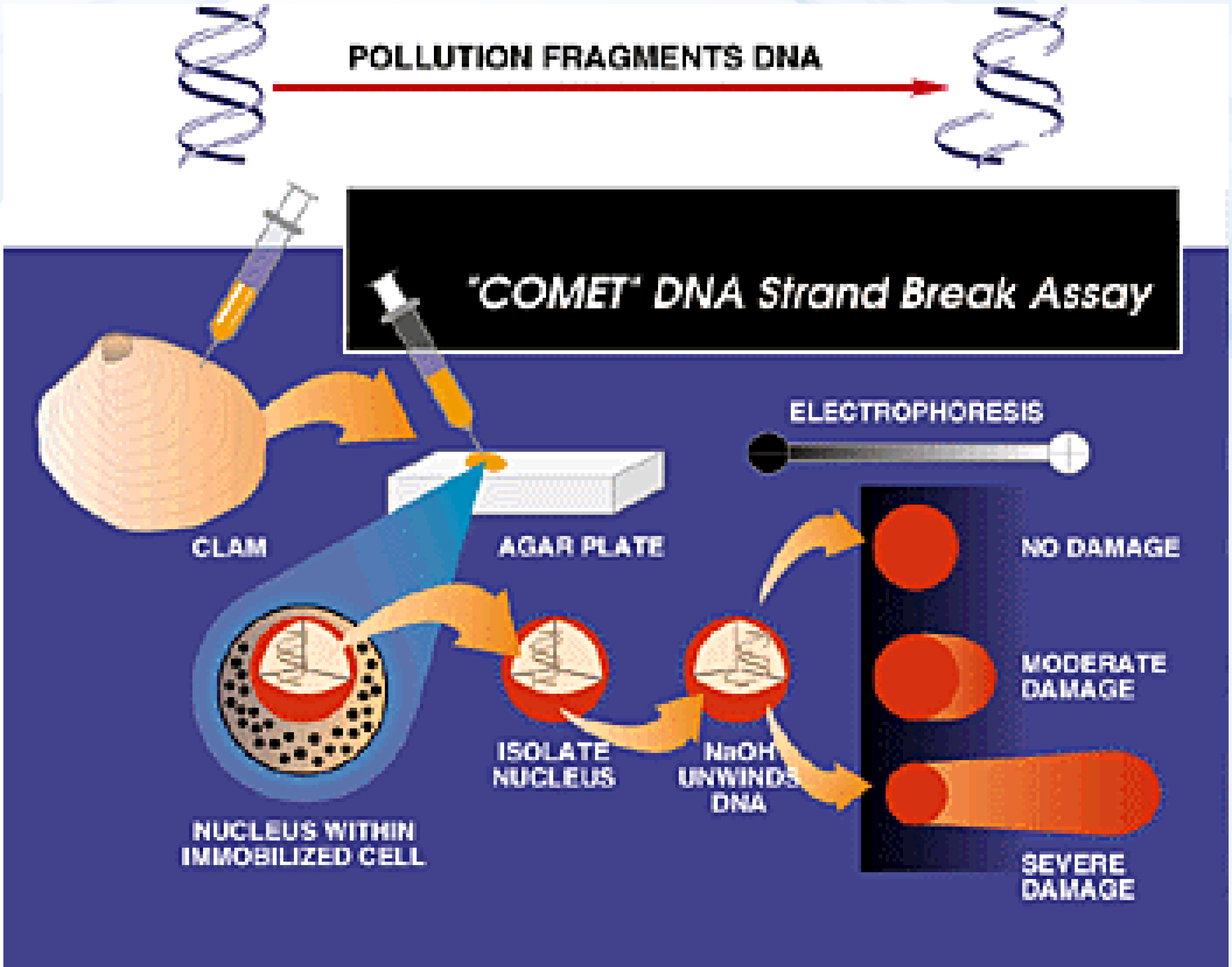
3.2 **chromosomal abnormalities**

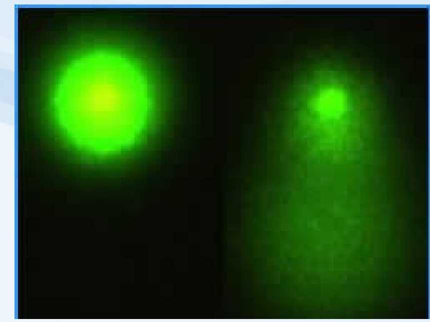
karyotype biomarkers (*human genetic disorders)*

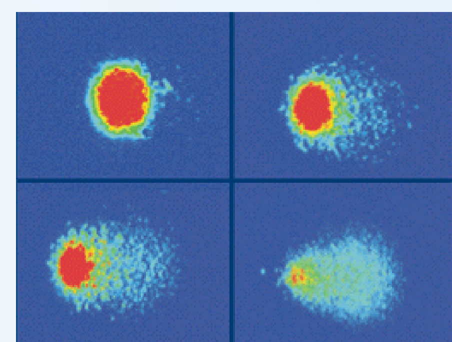


3) Nuclear DNA damage characterization

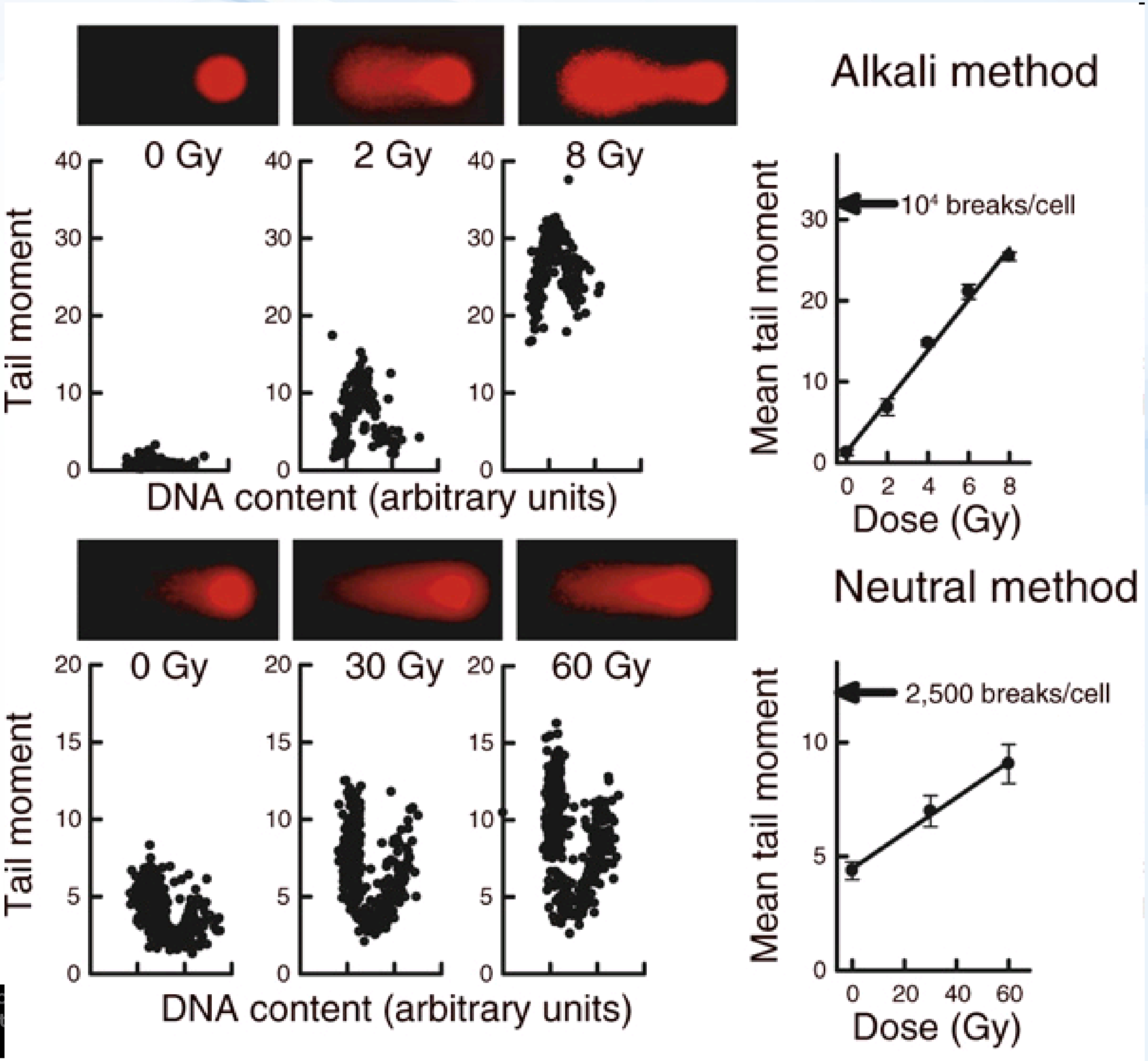
3.3.COMET ASSAY







Example results - Comet assay vs. radiation



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Standard clinical chemistry & hematology biomarkers

Clinical chemistry & hematology

Non-destructive (BLOOD, URINE sampling)

Multipe parameters can be measured

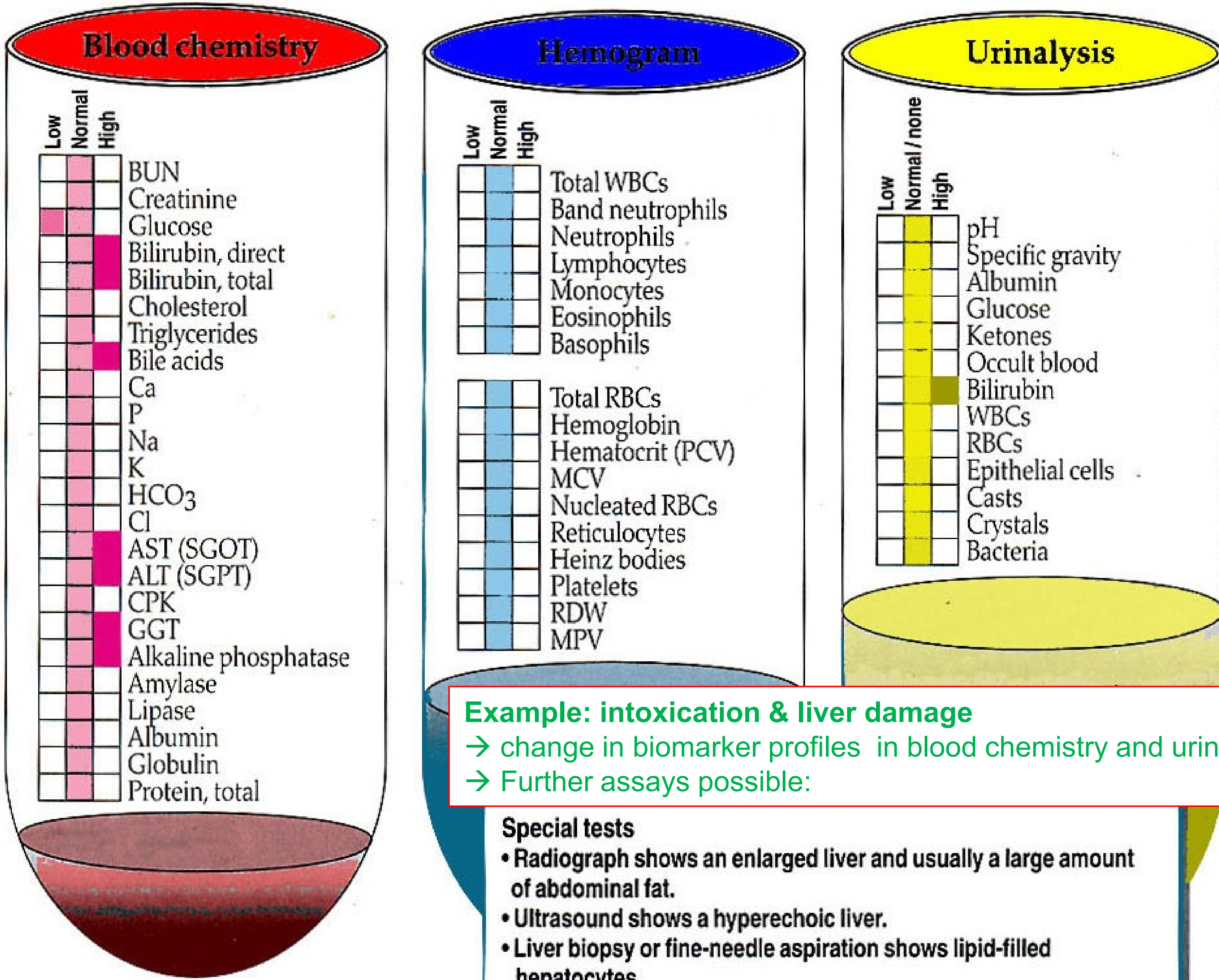
- responses to various types of stresses (including toxic stress)
- „normal“ value ranges known for humans, rats and few other species *(limited use as biomarkers in other organisms)*

Blood analyses

- **chemistry and biochemistry**
- **cells (hemogram)**

Urine analyses

- **chemistry, cells, bacteria etc.**



Example: intoxication & liver damage

→ change in biomarker profiles in blood chemistry and urine

→ Further assays possible:

Special tests

- **Radiograph shows an enlarged liver and usually a large amount of abdominal fat.**
- **Ultrasound shows a hyperechoic liver.**
- **Liver biopsy or fine-needle aspiration shows lipid-filled hepatocytes.**

Methods in clinical chemistry

Methods:

- automatic biochemical and hematological analyzers
- different „analytes”: various principles of methods (see example →)

Methods in clinical chemistry

Example

- determination of enzymatic activities in blood

- interpretation: tissue/organ-specific damage damage

Examples *(toxicological studies)*

- Liver damage (toxicants, POPs, alcohol)
  - **AST** (Aspartate aminotransferase),
  - **ALT** (Alanine aminotransferase) in blood
- General damage in cell (tissue non-specific)
  - **LDH** - lactate dehydrogenase
- Muscle damage:
  - **creatine kinase** in serum (isozymes - tissue specific – muscle vs heart);

Other enzyme biomarkers→ see further

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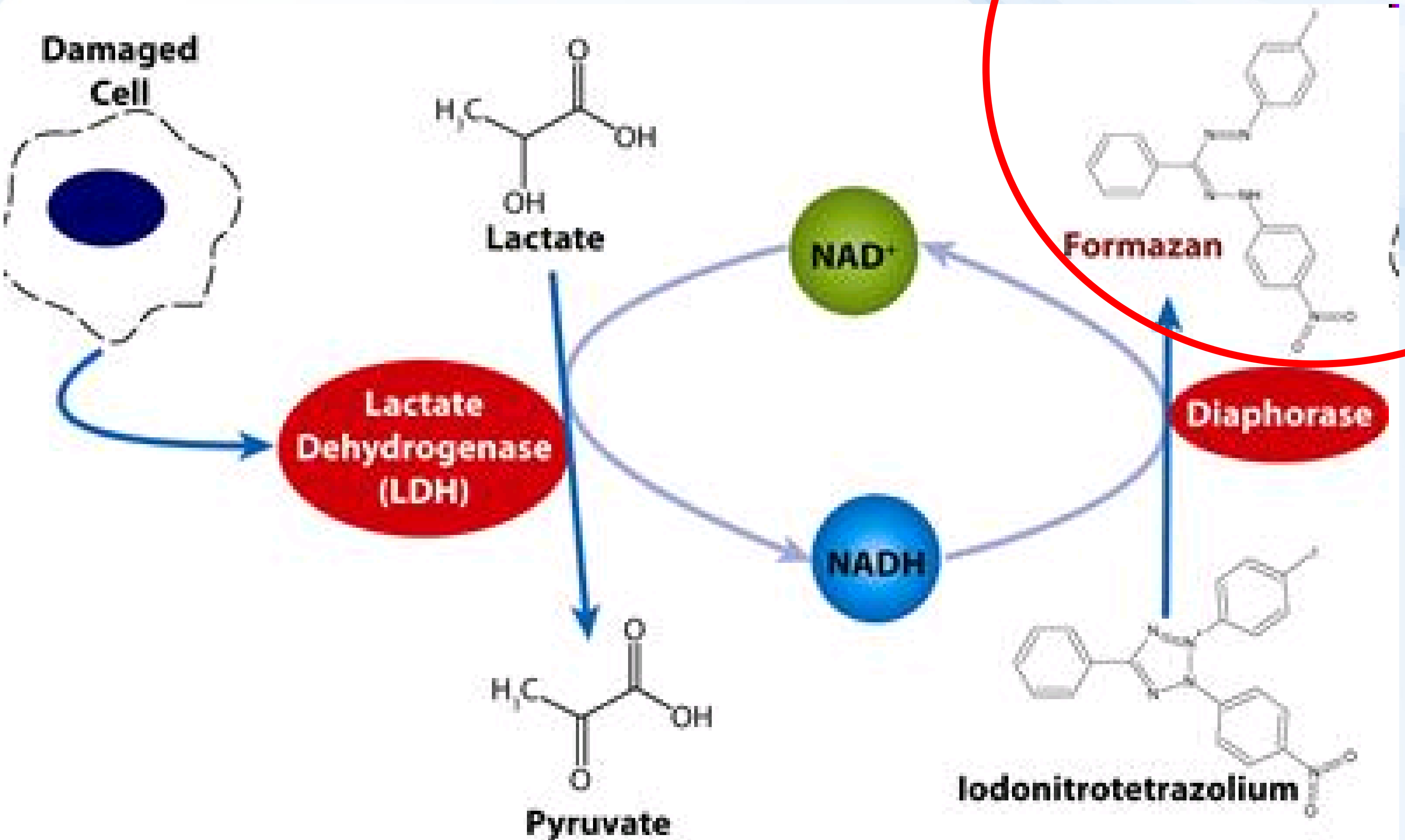
Methods in clinical chemistry: example LDH analysis

Done in automatic analyzer:

•Blood sample + addition of (NAD+ tetrazolium salt + diaphorase enzyme)
•Incubation and spectrophotometry determination
•Automatic evaluation → final value (LDH activity)
→ comparison with “limits” → highlighting for a doctor

Coloured product: kinetic spectrophotometry



Example – changes in rat serum enzymes after CCl_4 exposure

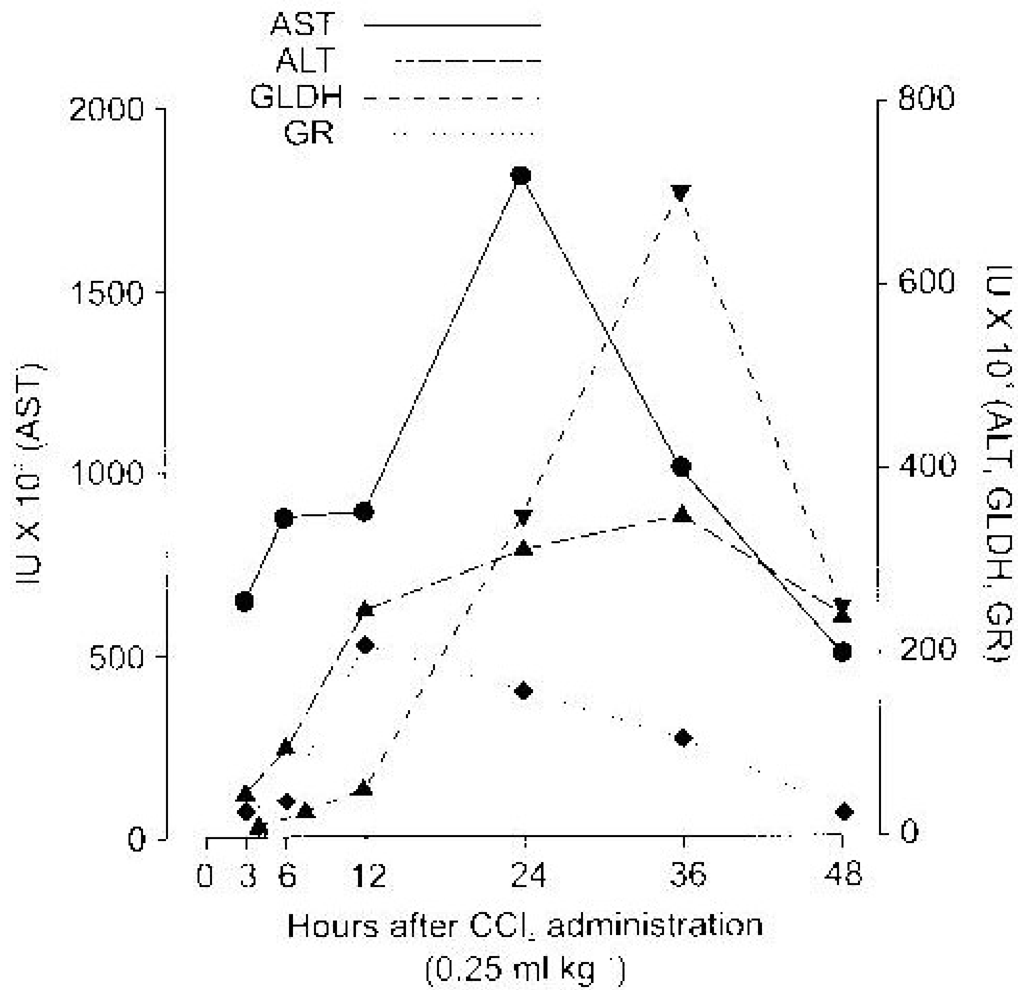


Figure 3 Serum enzyme levels in rats following dosing with carbon tetrachloride (CCl_4, 0.25 ml kg^{-1}). Redrawn from Zimmerman (1978).

Cell damage (Liver) enzyme activity (LDH) is also highly variable and species-specific

Table 6.2 Effects of pollutants on LDH

PHAHs		
DDE	+ Quail	Dieter (1974)
	+ Starling	Dieter (1975)
DDT	= Redstart	Karlsson *et al.* (1974)
PCBs	= Redstart	
	+ Quail	Dieter (1974)
	+ Starling	Dieter (1975)
Endrin	− Fish (*Ophiocephalus*)	Sharma *et al.* (1979)
Photomirex	+ Rat	Chu *et al.* (1981)
OPs		
Malathion	+ Rat	Dragomirescu *et al.* (1975)
	+ Quail	Dieter (1974)
	+ Starling	Dieter (1975)
	− Carp	Dragomirescu *et al.* (1975)
Methylparathion	+ Chicken	Somlyay *et al.* (1989)
Phosmethylan	+ Chicken	
Methidathion	+ Carp	Asztalos *et al.* (1990)
Metals		
Cadmium chloride	= Brook trout	Christensen *et al.* (1977)
Copper sulphate	+ Carp	Dragomirescu *et al.* (1975)
Lead nitrate	= Brook trout	Christensen *et al.* (1977)
Mercuric chloride	+ Quail	Dieter (1974)
	= Brook trout	Christensen *et al.* (1977)
	+ Fish (*Notopterus*)	Verma and Chand (1986)
Methylmercury	+ Starling	Dieter (1975)
Others		
Oil	= Striped mullet	Chambers *et al.* (1979)
Paraquat	+ Carp	Asztalos *et al.* (1990)

Biomarkers:
Changes in enzyme activities

Enzymatic changes

Biomarkers reflecting „enzyme changes“:

EXAMPLES - inhibitions of specific enzymes
(as also discussed earlier during the class: MoA)
AcChE (organo-phosphates)
Proteinphosphatases (microcystins)

(+) Rapid enzymatic assays, specific responses
(-) Some ~ EXPOSURE biomarkers

Reminder: AcChE inhibition mechanism

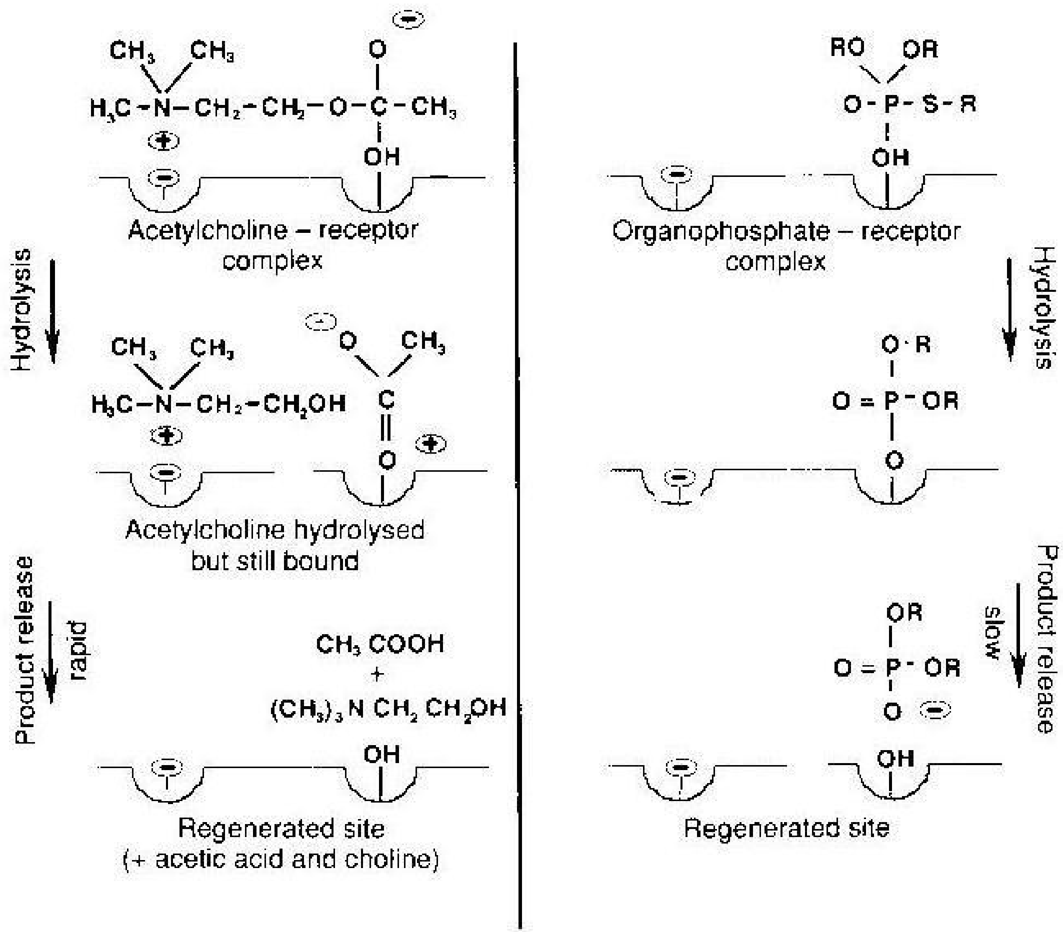


Figure 2.2 Mode of action of inhibition of acetylcholinesterase.

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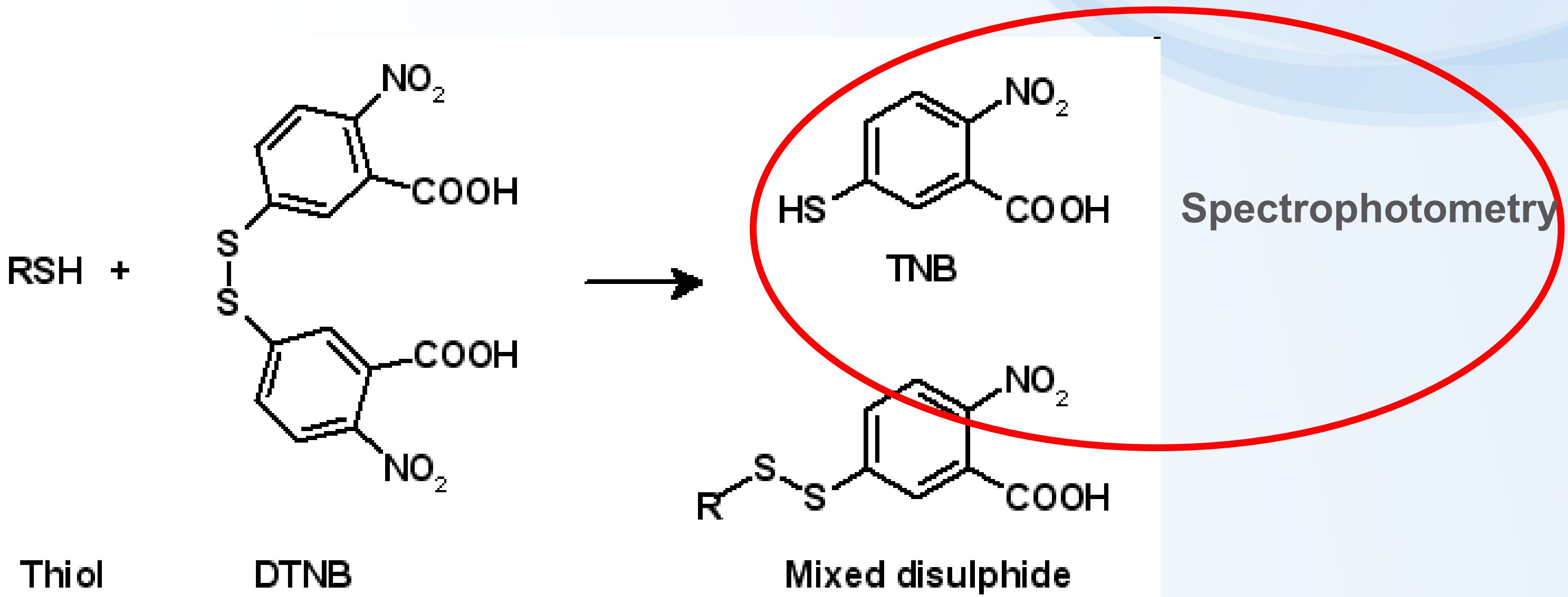
AcChE assessment

Model Substrate (butyryl-thio-choline, acetyl-thio-choline)

- cleaved by **AcChE** → formation of free –SH groups
- reaction of SH with **thiol reactive probe** = **Ellman´s reagent (DTNB)**

→ DTNB-S-choline: yellow colour (spectrophotometry A420)



Changes in AcChE in birds after exposure to organophosphates

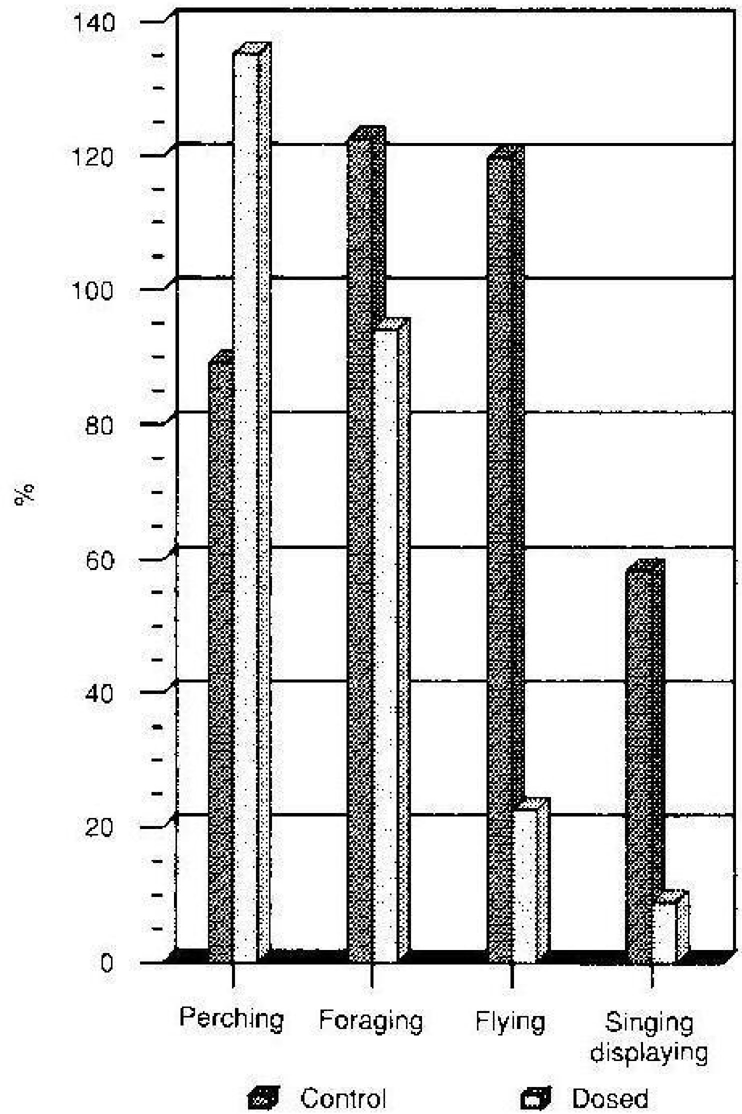


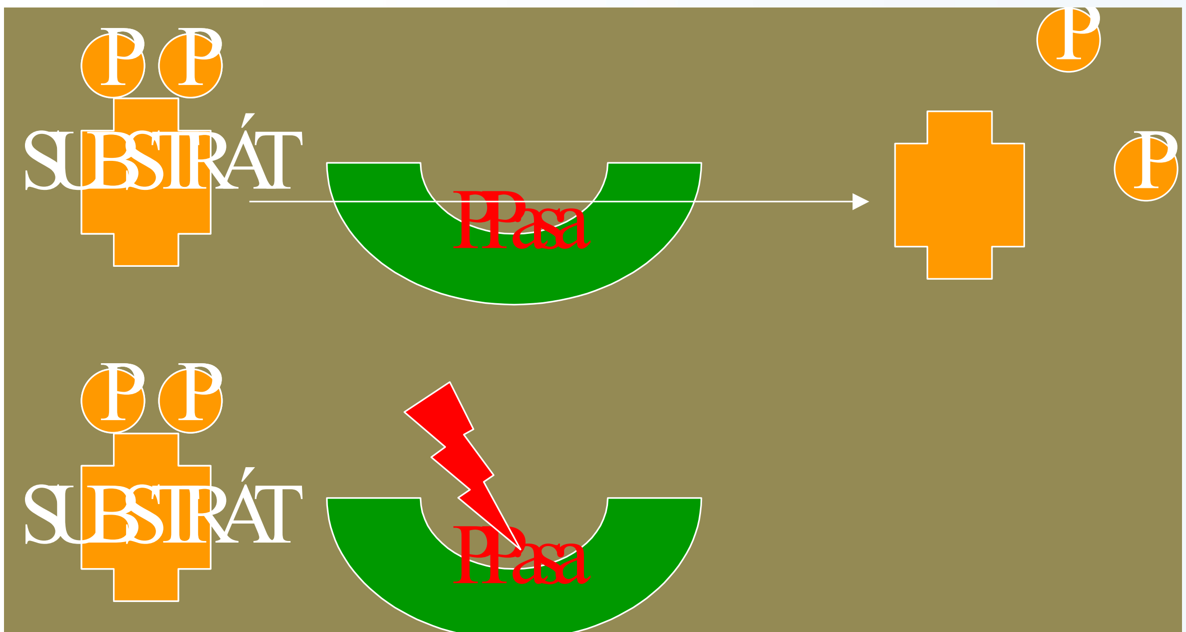
Figure 2.4 Effect of OP on behaviour of starlings. After Grue and Shipley (1981).

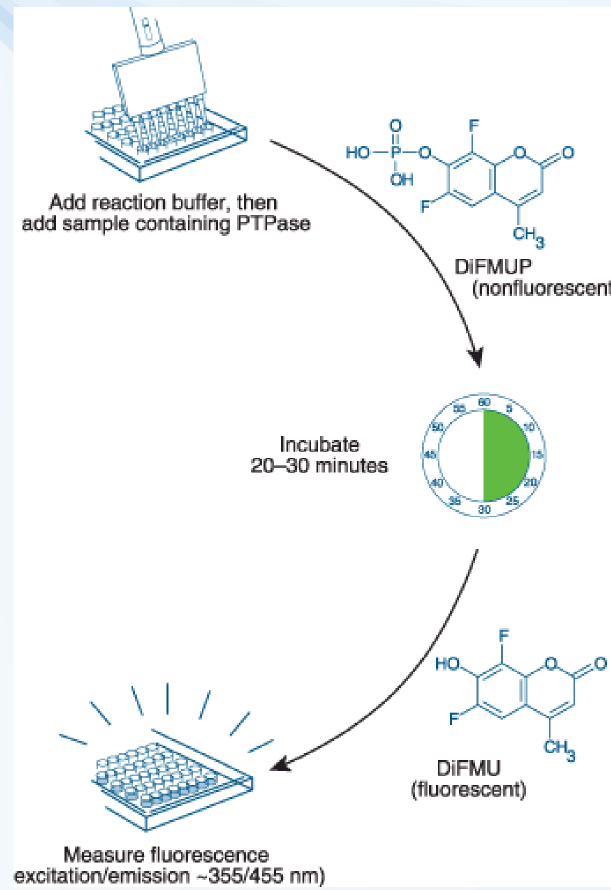
Proteinphosphatase (PPase) inhibition assay

Model substrates cleaved by PPase

^{32}P-labelled protein → free **^{32}P radioactivity**

6,8-difluoro-4-methylumbelliferyl phosphate → **fluorescence**





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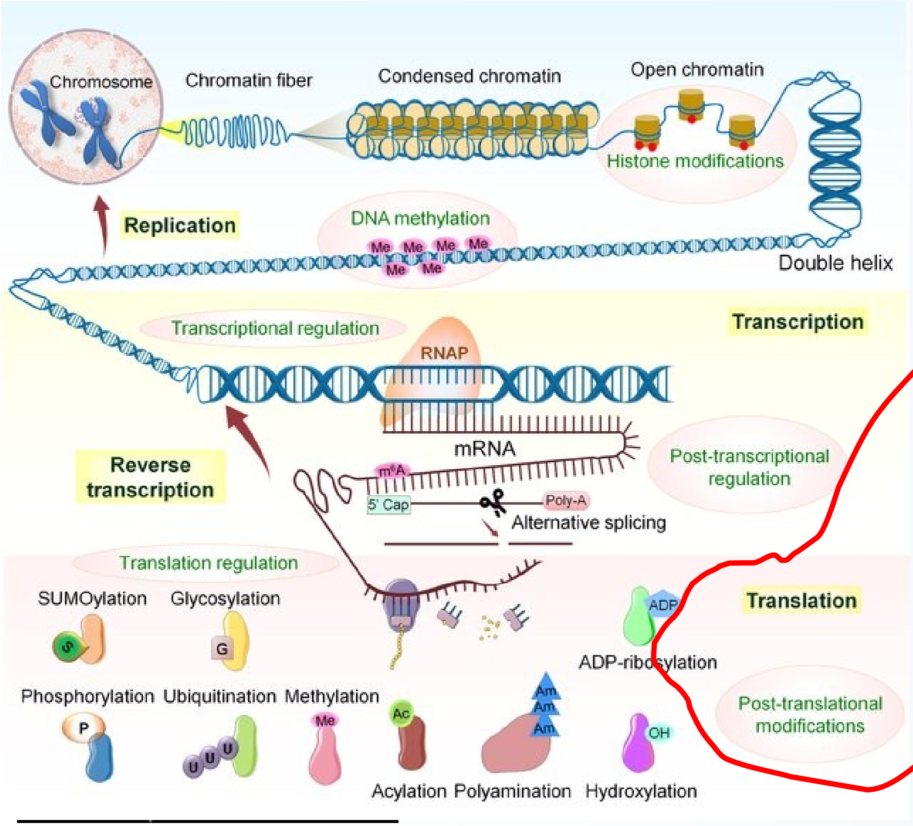
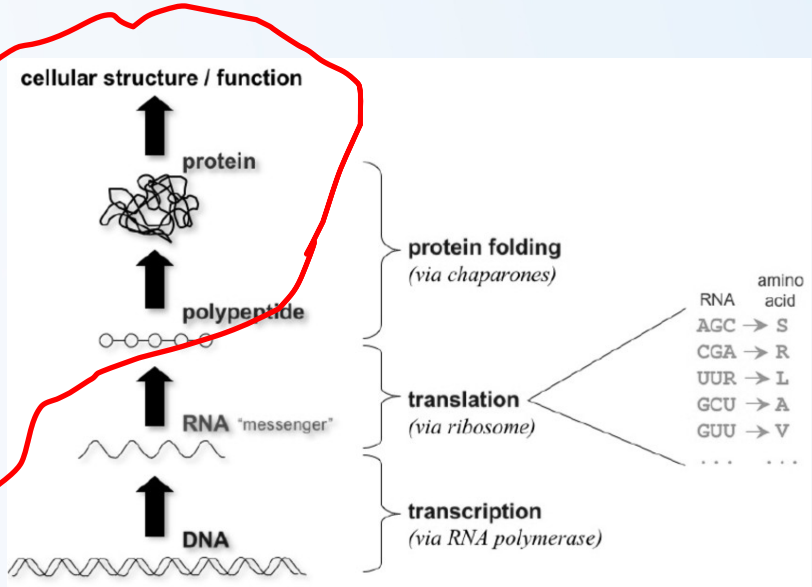
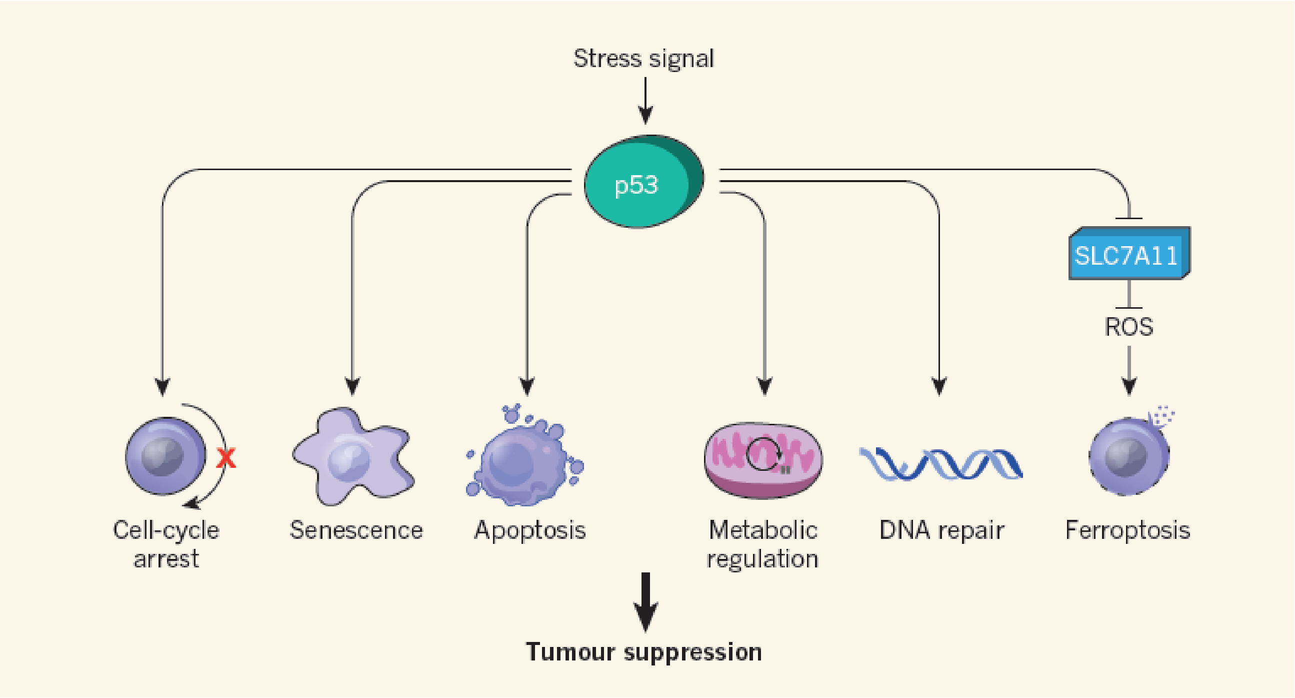
Biomarkers – assessing gene and protein expressions / levels





Protein modulation: toxic response at several levels



Toxicants induce various changes in the cell …

… many of these changes result in

→ activation / deactivation of specific genes

→ modulated gene expression

→ modulated protein levels

... and protein activities

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Nature (2015) vol 520, p. 37

How to measure gene and protein modulations?

Traditional methods of QUANTIFICATION at different levels

- **mRNA levels**
  - PCR / quantitative RT-PCR
- **protein levels**
  - electrophoresis and Western-(immuno)blotting
  - ELISA techniques
- **induced protein enzymatic activities** associated with elevated protein levels
  - eg. enzymatic activity

New types of complex techniques: “omics” → also will be discussed later

Examples of targeted protein biomarkers – discussed further →

specific protein markers of disease / e.g. cancer

heat shock proteins (hsp90, hsp60, hsp 70, ubiquitin)

metalothioneins

endocrine disruption biomarkers
- Vitellogenin(-like) Vtg proteins in male
- Aromatase

Induction of detoxification enzymes
- CYP450 / EROD
- GST

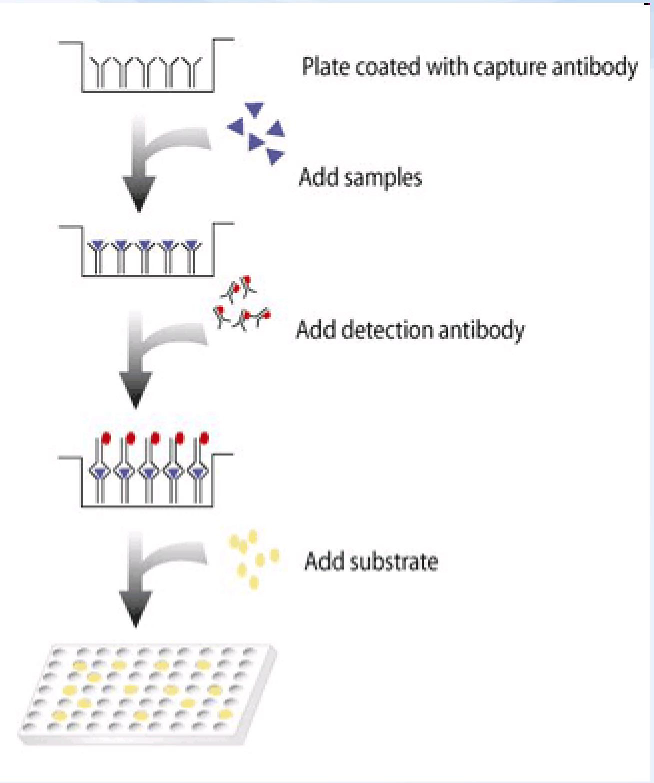
Tumor genes and tumor markers

- cancer genes *ras, myc – e.g.* ***metastasing bowel cancer***
- α*-fetoprotein (AFP) – elevated during fetus development AND e.g. liver cancers*
- tumor suppressor genes (e.g. *p53) – indicate better prognosis for certain cancers*
- *PSA – prostate-specific antigen:* ***prostate cancer*** *in males (over 50 years of age)*

Methods of determination in practice:

ELISA

(enzyme linked immunosorbent assays)



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Heat Shock Proteins (hsp)

General stress = synthesis of new proteins

~ equilibrium and homeostasis buffering

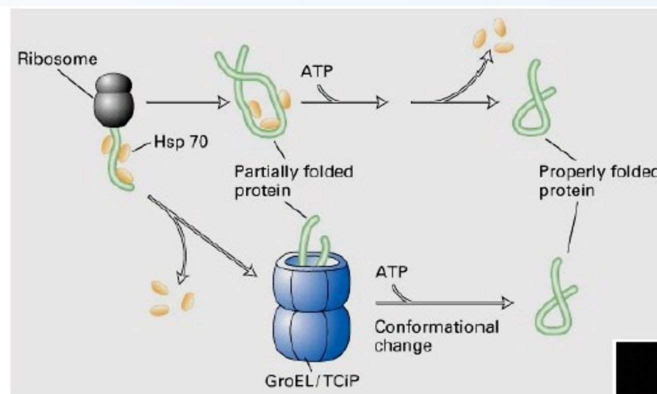
- temperature (cold / heat) → proteins assuring cryo-preservation
- salinity & metals → ion buffering
- organic xenobiotics → detoxication

New proteins must be folded to their 3D stucture

by activity of **„CHAPERONES“**

Chaperons = hsp90, hsp60, hsp 70

~ 60-90 kD molecular weight kD



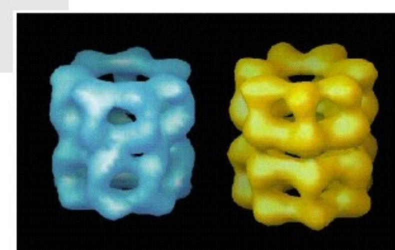


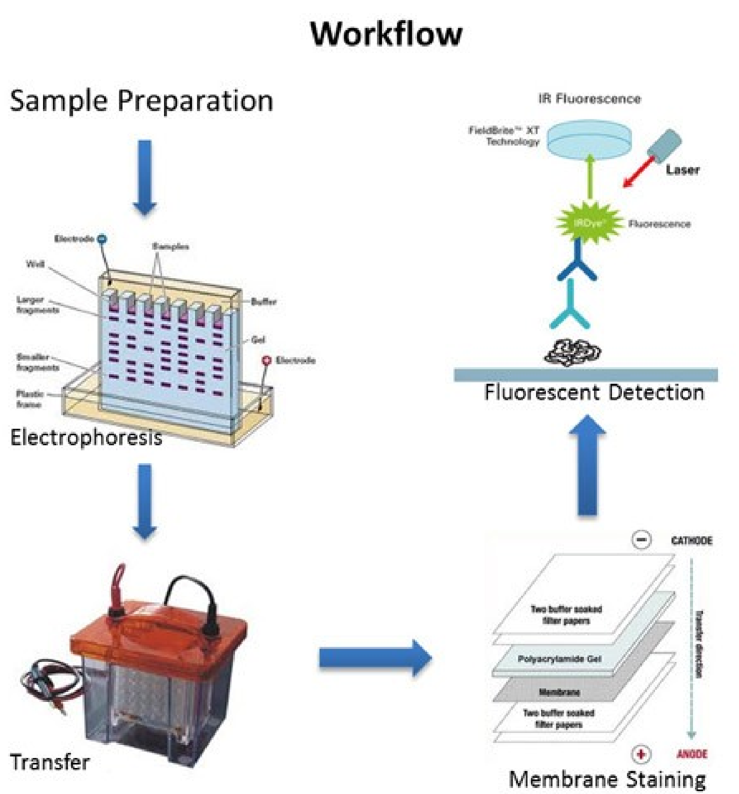
Figure 3-15

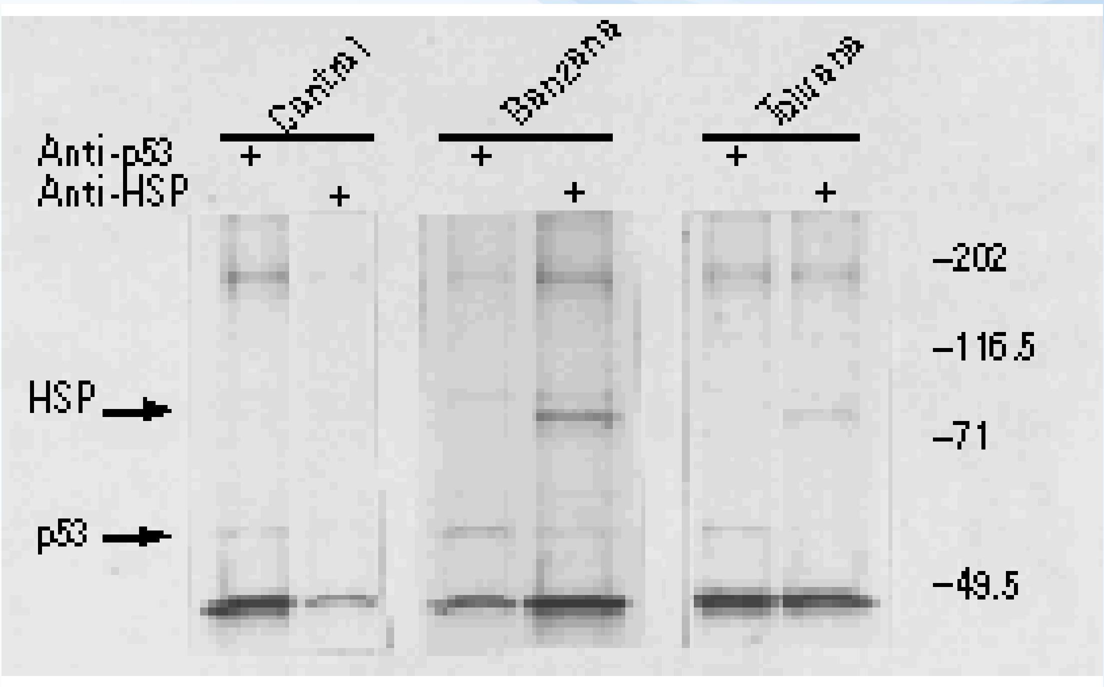
HSP determination - example

HSP = GENERAL STRESS biomarker, non-specific

- phylogenetically conserved (similar genes in most of the organisms)
- structural similarity → easy determination:

  electrophoresis + immunoblotting (**Western blotting**)





Metalothioneins (MTs, MT-like proteins)

Low MW proteins (6-10 kD) rich of Cystein (-SH)

- detected in numerous eukaryotic organisms
- induced in the presence of metals or less specific stress (low O2, T)
- long halflife (~ 25 days)
- binding of divalent metals (Zn, Cd, Hg) → exposure elimination
- natural function (?) – regulation of essencial metals in cells

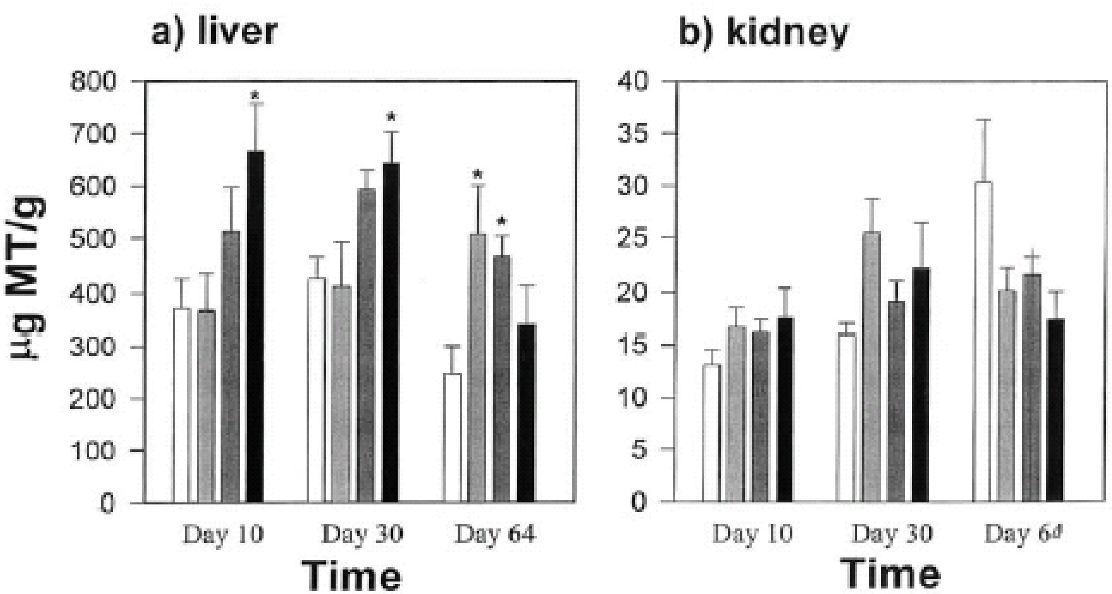


Fig. 2. Metallothionein (MT) concentrations in the (a) livers and (b) kidneys of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (±S.E.). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$). See Fig. 1 for an explanation of histogram shading.

Protein biomarkers of estrogenicity / ED-like effects

ER = transcription factor controling number of target genes

Target genes of ER = biomarkers of estrogenicity

Major examples

- **Vitellogenin**
- **Aromatase - CYP19A**

Vitellogenin (Vtg)

Precursor of yolk proteins, phospho-protein („energy“ rich)

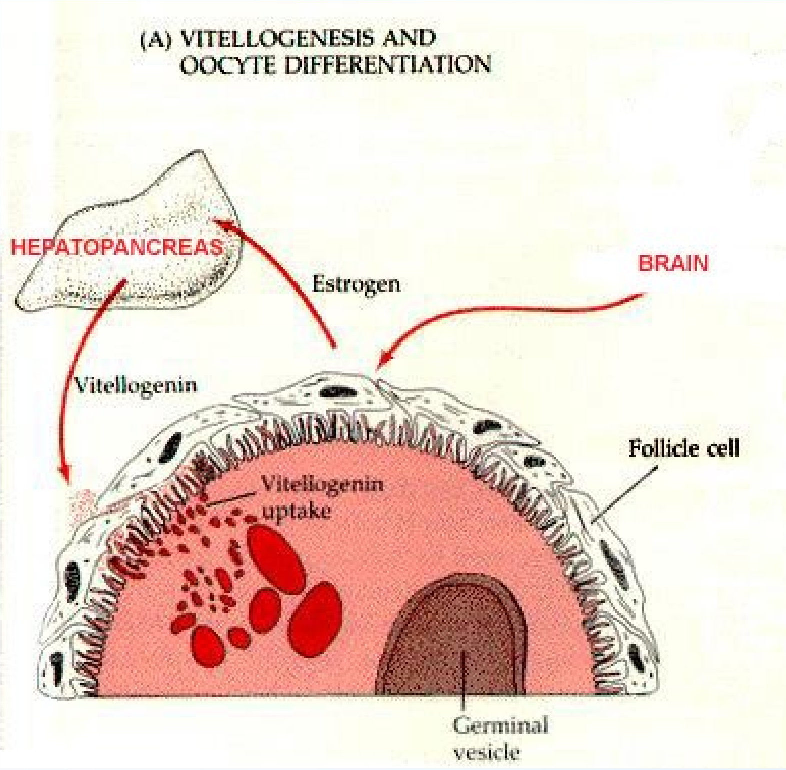
→ egg formations (females) **at oviparous vertebrates**

Synthesized in liver and distributed via blood / haemolymph

Xenoestrogens & other endocrine disruptors

→ increased levels or early production in FEMALES

→ production de novo in **MALES**



Vitellogenin (Vtg) assessment

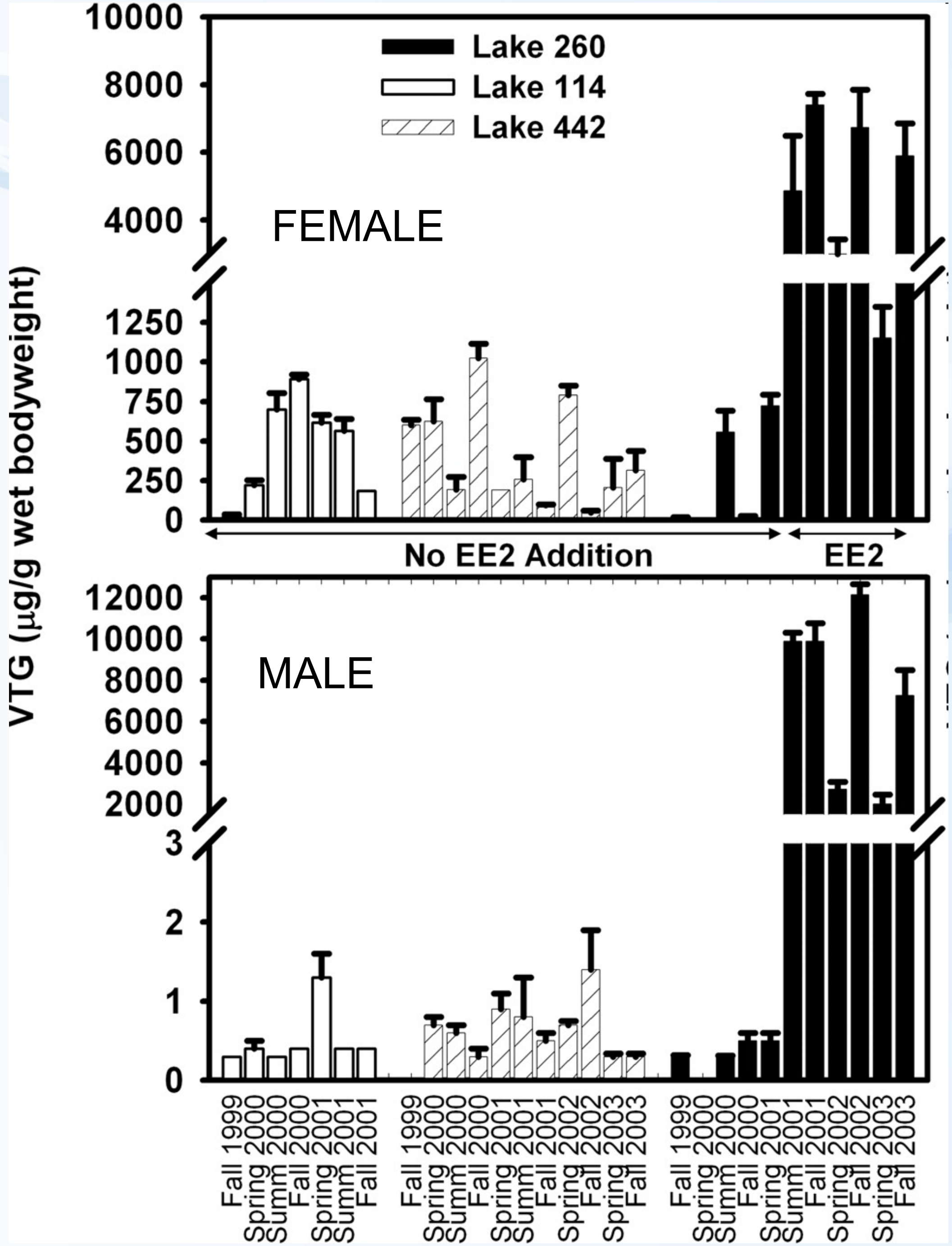
1) ELISA in exposed organisms (F/M) or in vitro

(-) specific antibodies are necessary for each species
(low crossreactivity of Abs)

2) „Vitelin-like proteins“

- total amount of „alkali-labile“ phosphate in haemolymph (mussels)
- alkaline extraction of P from sample → spectrophotometric determination

Vitellogenin in fish



Kidd et al. (2007) PNAS

Fig. 1. Mean SE (*n* = 4–7) VTG concentrations in whole-body homogenates of male (*Lower*) and female (*Upper*) fathead minnow captured in 1999–2003 from reference Lakes 114 and 442 and from Lake 260 before and during additions of 5–6 ng·L^{-1} of EE2 (low catches of fish in Lake 260 in 2004 and 2005 did not allow for these analyses in the latter 2 years of the study).

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Vitelin-like proteins in mussels

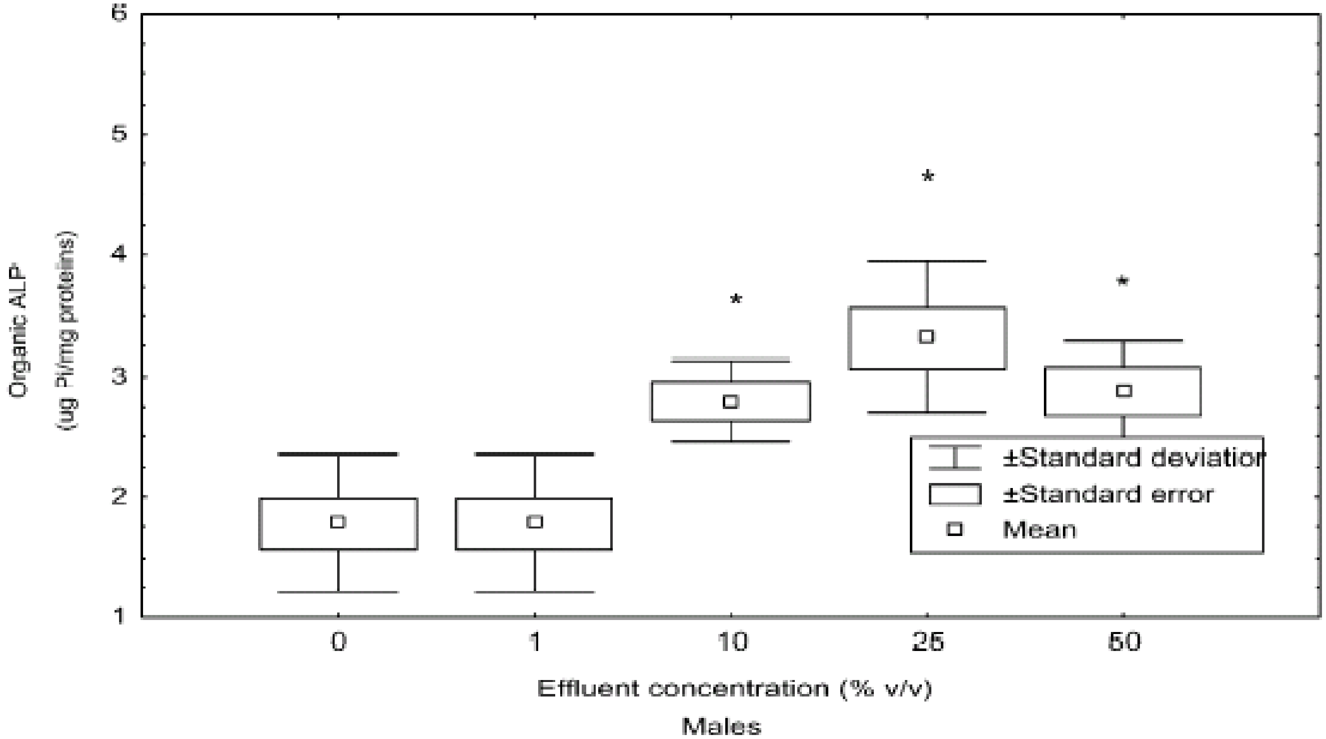
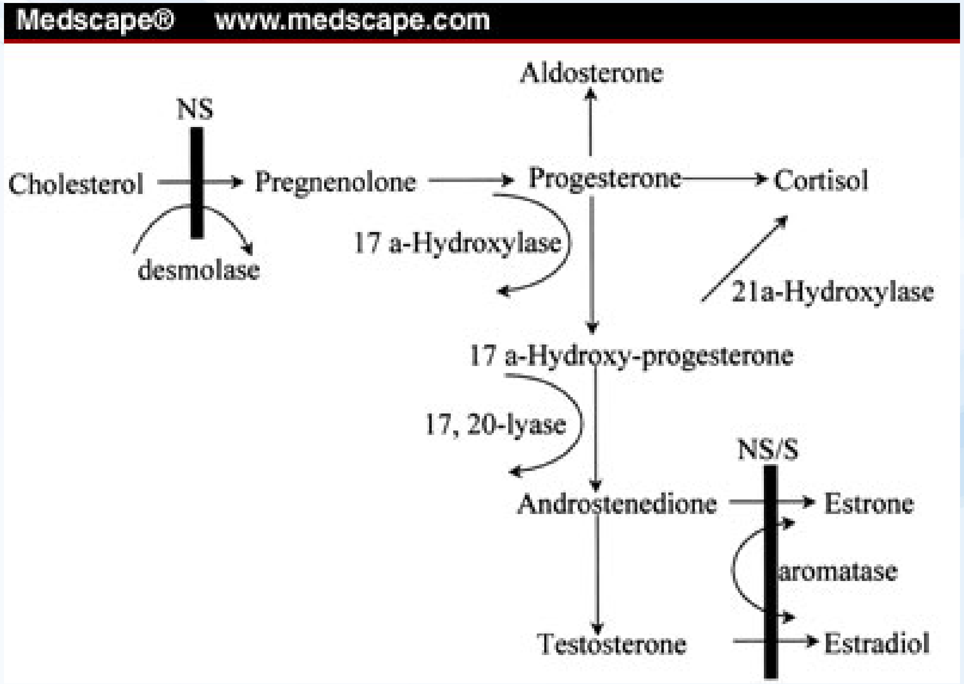


Fig. 4. Induction of Vg by exposure to a municipal effluent. Mussels were exposed for 96 h to a municipal effluent at 15°C. They were then collected for Vg and sex determinations. The asterisk (*) indicates significant difference at $P < 0.05$.

Aromatase (CYP19A)

- Levels inducible by estrogens
- Catalyzes single enzymatic step

androgens → estrogens



Experimental assessment - mRNA
(in reseach and practice)

1. PCR / Quantitative-Real-Time-PCR

2. GM-organisms (zebrafish): reporter gene with GFP
   Green Fluorescence Protein under the control of aromatase promoter

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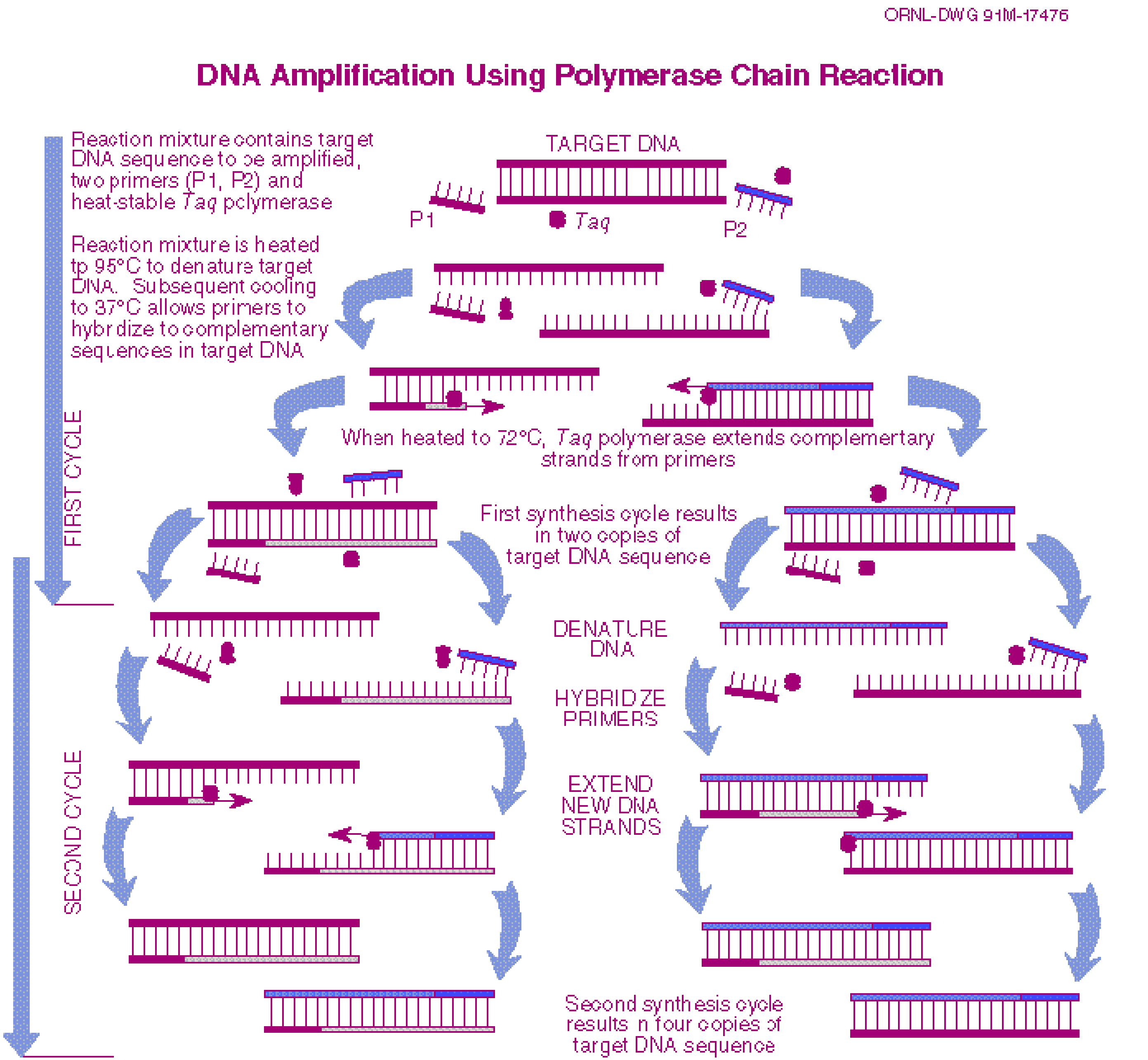
PCR principle



Source: *DNA Science*, see Fig. 13.

Visualization of PCR product

1) Electrophoresis (qualitative)

Intercalation dyes

– e.g. **ethidium bromide**

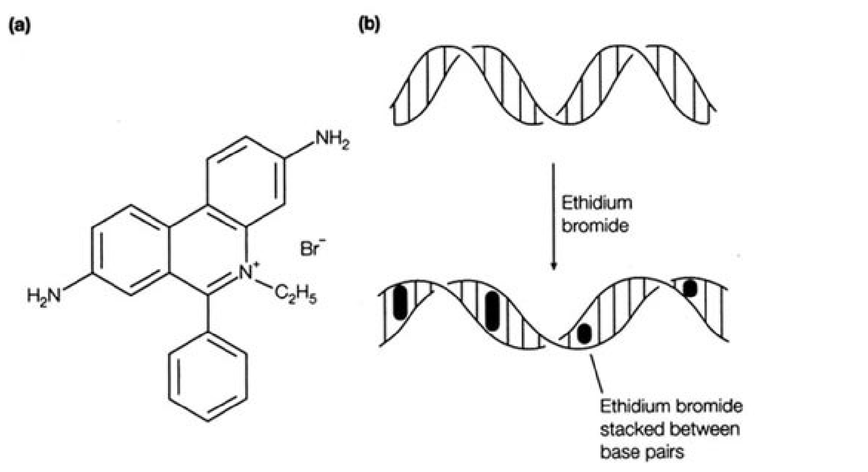
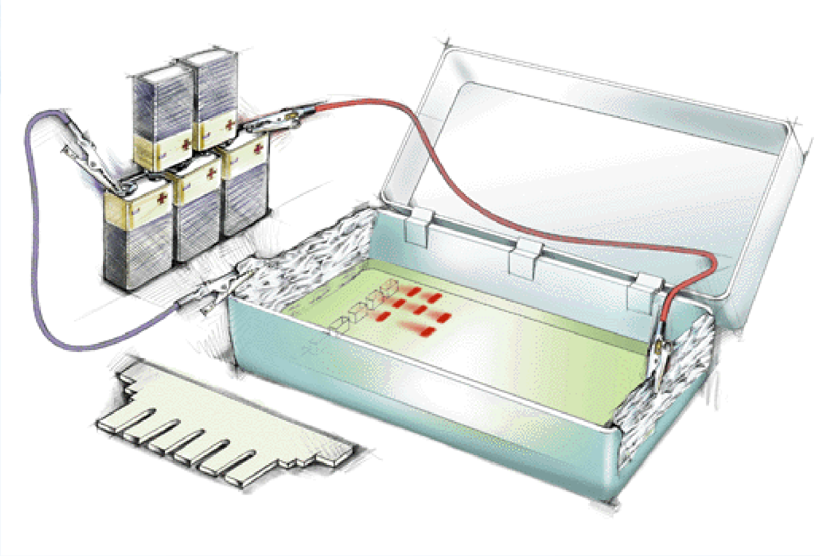
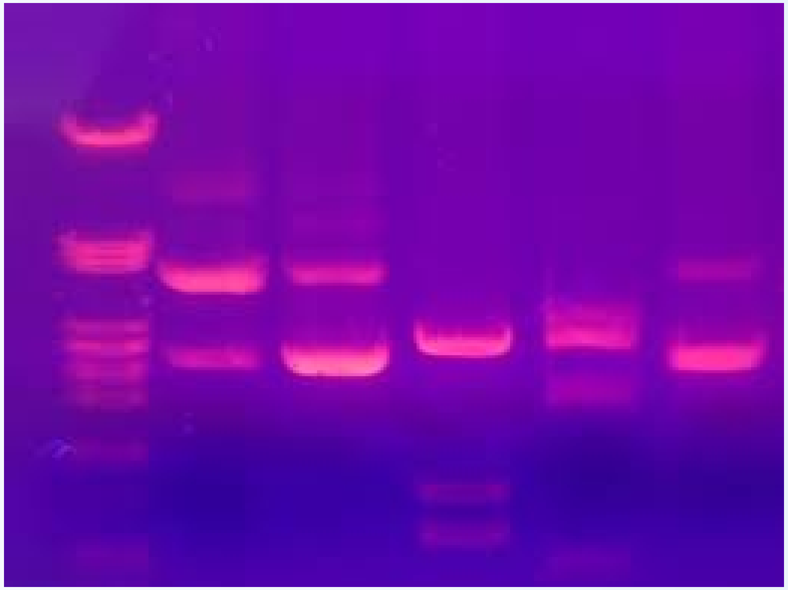


Fig. 3. (a) Ethidium bromide; (b) the process of intercalation, illustrating the lengthening and untwisting of the DNA helix.

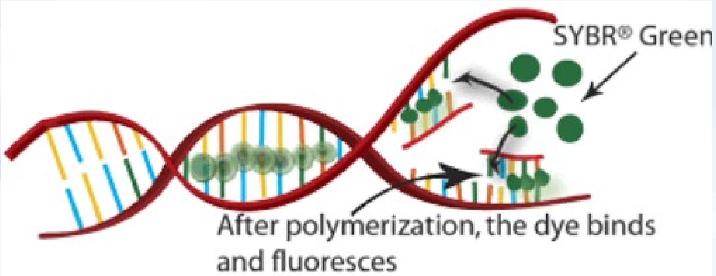


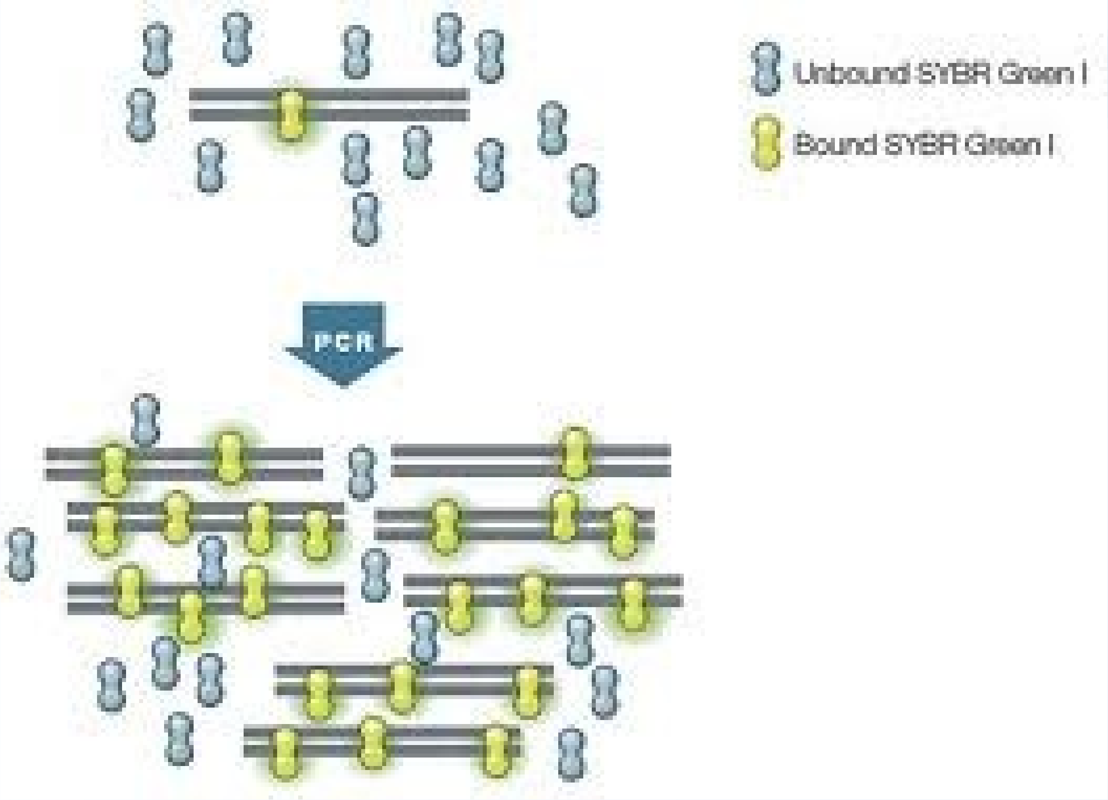


Visualization of PCR product

2a) Real-time (quantitative) SYBR GREEN dye

→ more DNA synthesized,
more fluorescent dye incorporated
→ **Higher fluorescence**



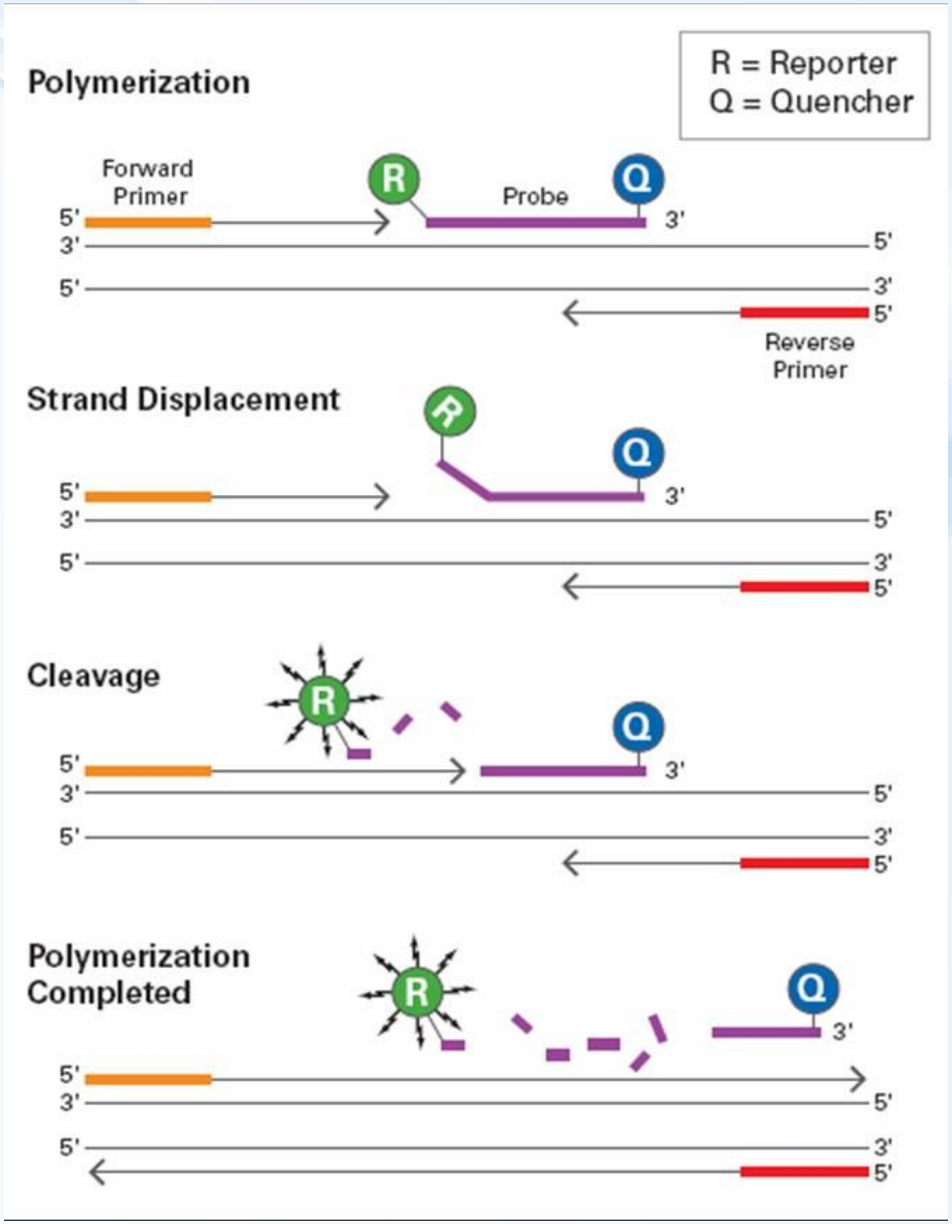


Centrum pro výzkum toxických látek v prostředí

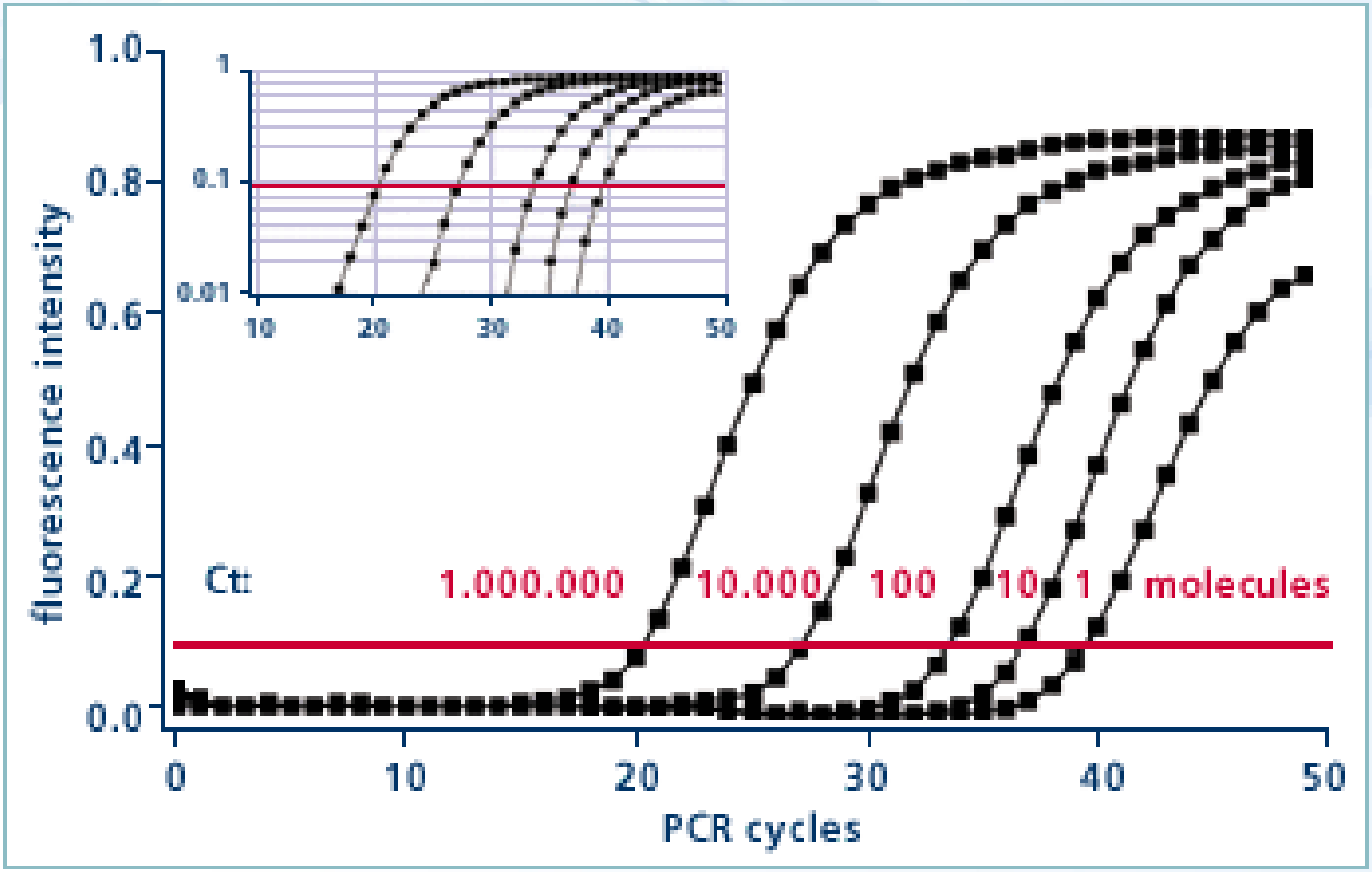
Visualization of PCR product

2b) Real-time (quantitative) TaqMan probes

(more DNA replications
more fluorescent dye released)



“Quantitative” determination of PCR product



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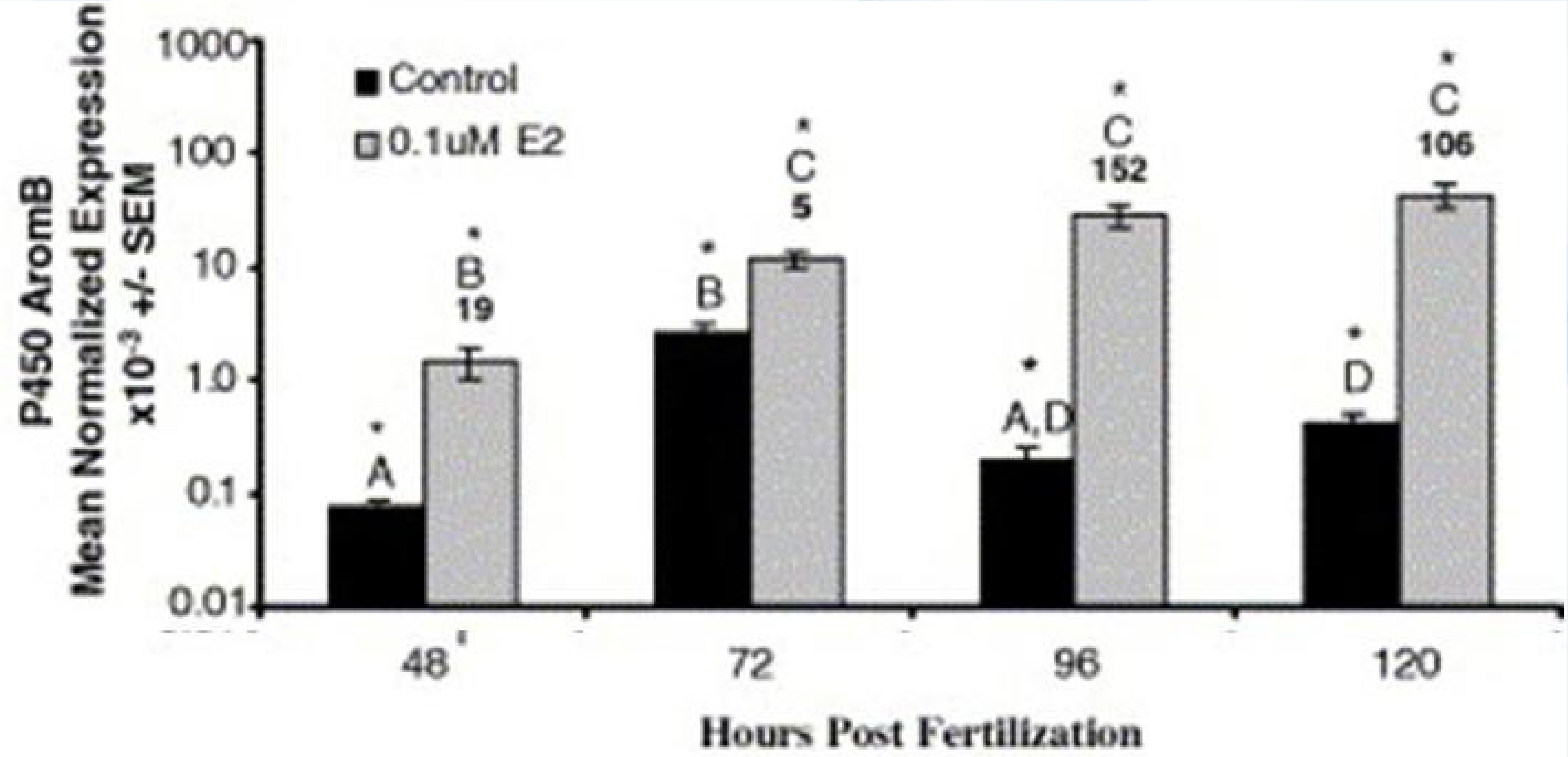
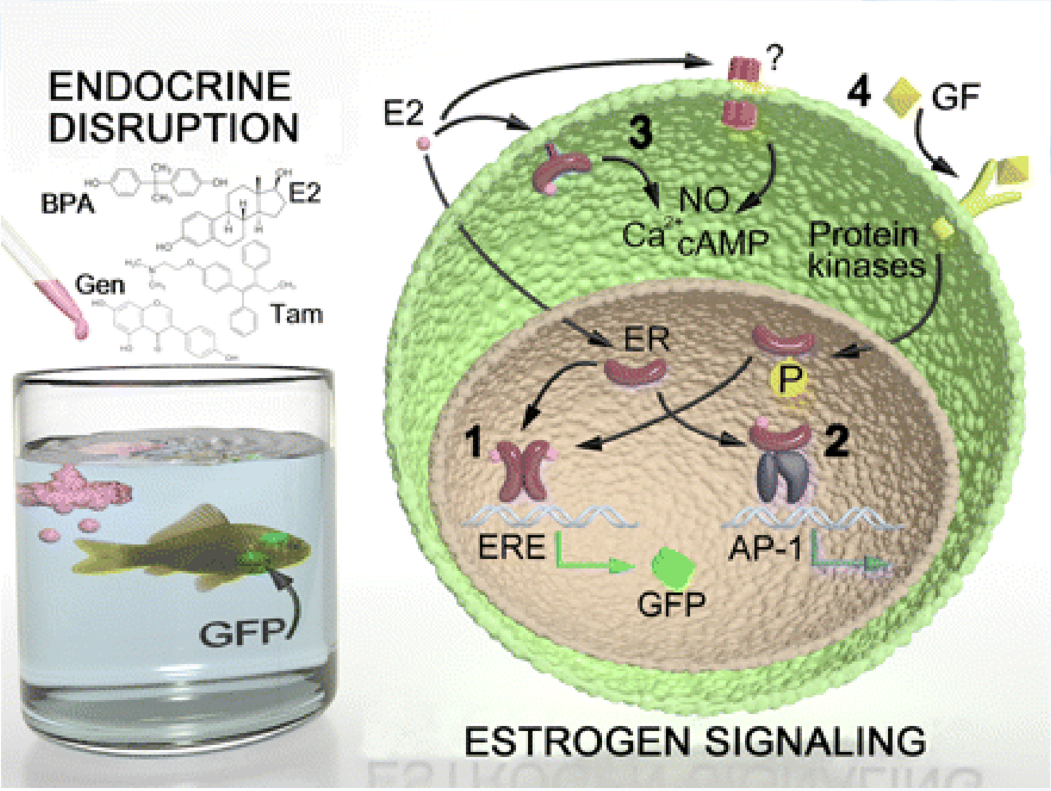
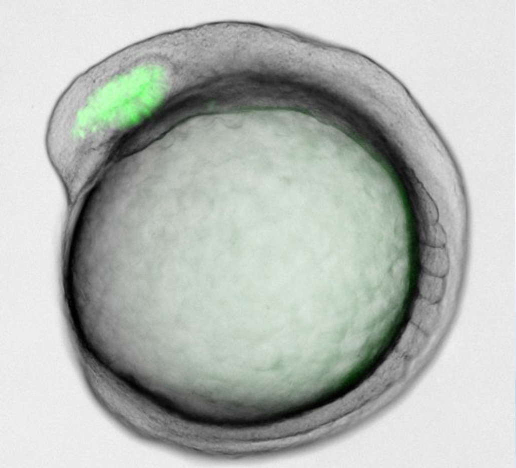
qPCR determination of the aromatase gene in Zebrafish

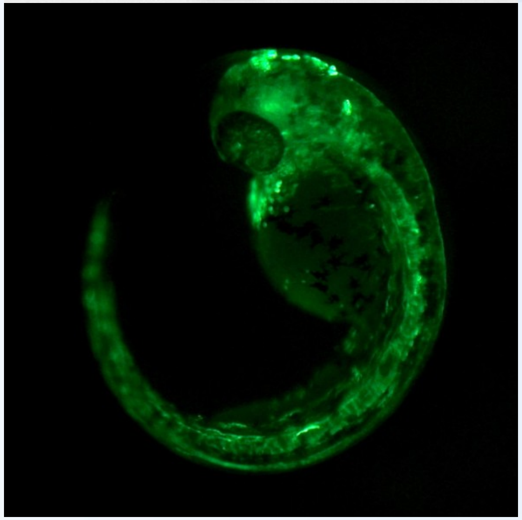


http://dx.doi.org/10.1016/j.ygcen.2005.12.010,

GFP-reporter for estrogens in zebrafish embryo







http://endo.endojournals.org/content/152/7/2542.full

Centrum pro výzkum toxických látek v prostředí

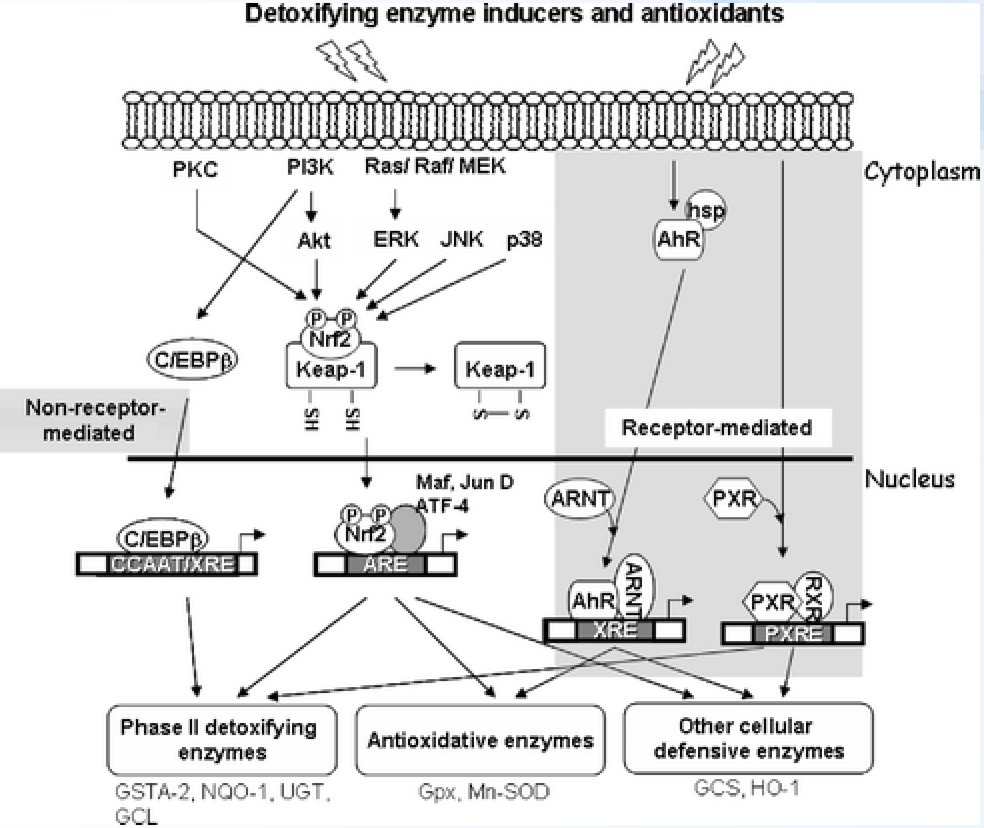
Inductions of detoxication & oxidative stress enzymes

(hepatopancreas / liver / blood)

MFO - CYP classes - **EROD** / MROD / BROD

Phase II enzymes (GSTs)

Glutathion metabolism enzymes (GPx, GRs)



MFO (CYPs) - reminder

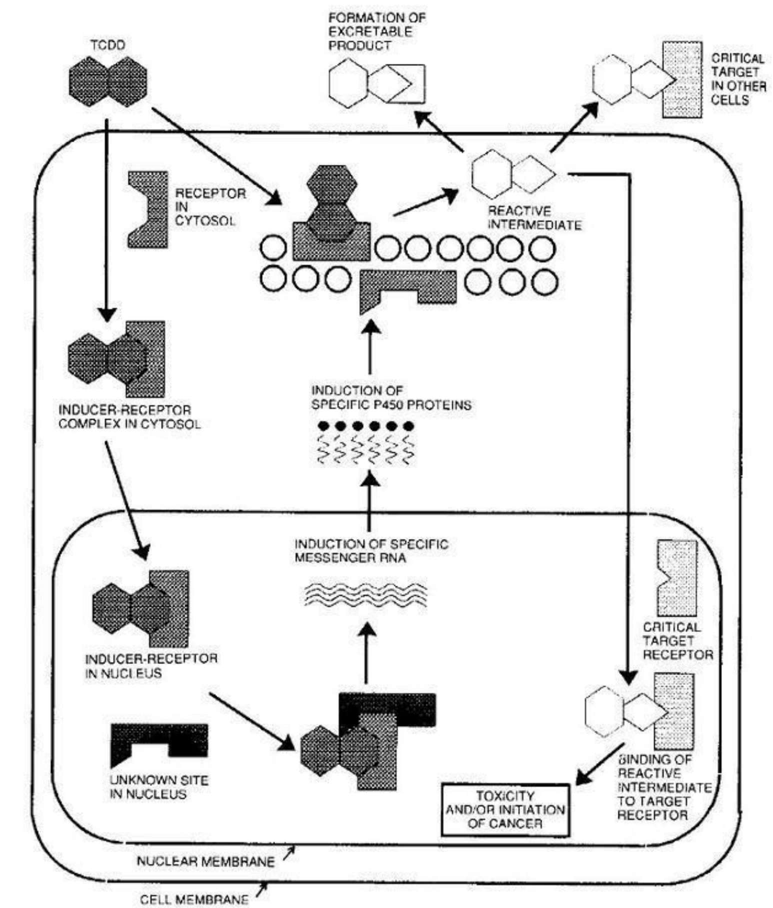


Figure 5.1 Diagram of MFO system. Nebert and Gonzalez (1987).

Table 5.1 Classification of P450s

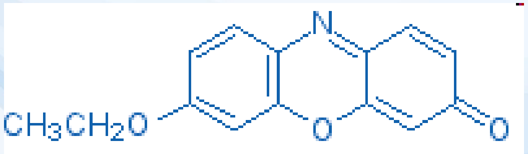
Nomenclature	Induced by/specificity
P450I	Polycyclic aromatic, TCDD
P450II	Phenobarbital-inducible family*
P450IIA	Specific for testosterone hydroxylase
P450IIB	PB inducible
P450IIC	PB inducible
P450IID	Specific for debrisoquine 4-hydroxylase
P450IIE	Ethanol inducible
P450III	Steroid inducible
P450IV	Specific to lauric acid w-hydroxylation
P450XI	Located in mitochondrion
P450XIA	
P450XIB	
P450XVII	Formation of steroid 17-hydroxylases
P450XIX	Involved in synthesis of oestrogens
P450XXI	Formation of steroid 21-hydroxylases
P450LI	Plant/yeast
P450CI	Prokaryote

* PB-inducible genes largely confined to P450IIB and C.
After Nebert and Gonzalez (1987).

Assessment of CYPs (MFO) – “EROD”

Determination of CYP1A1 activity

“EROD” - EthoxyResorufin-O-Deethylase activity



Substrate: **Ethoxyresorufin**

: Oxidation by CYP1A1 (MFOs)

→ Fluorescence (easy determination)

EROD = sensitive biomarker of organic pollution (exposure & effects)

: AhR-activating compounds (PCDD/Fs, PCBs, PAHs)

: often used in environmental studies

Use of other substrates: assessment of other CYPs

BROD – butoxy-ROD (CYP3A), MROD, PROD …

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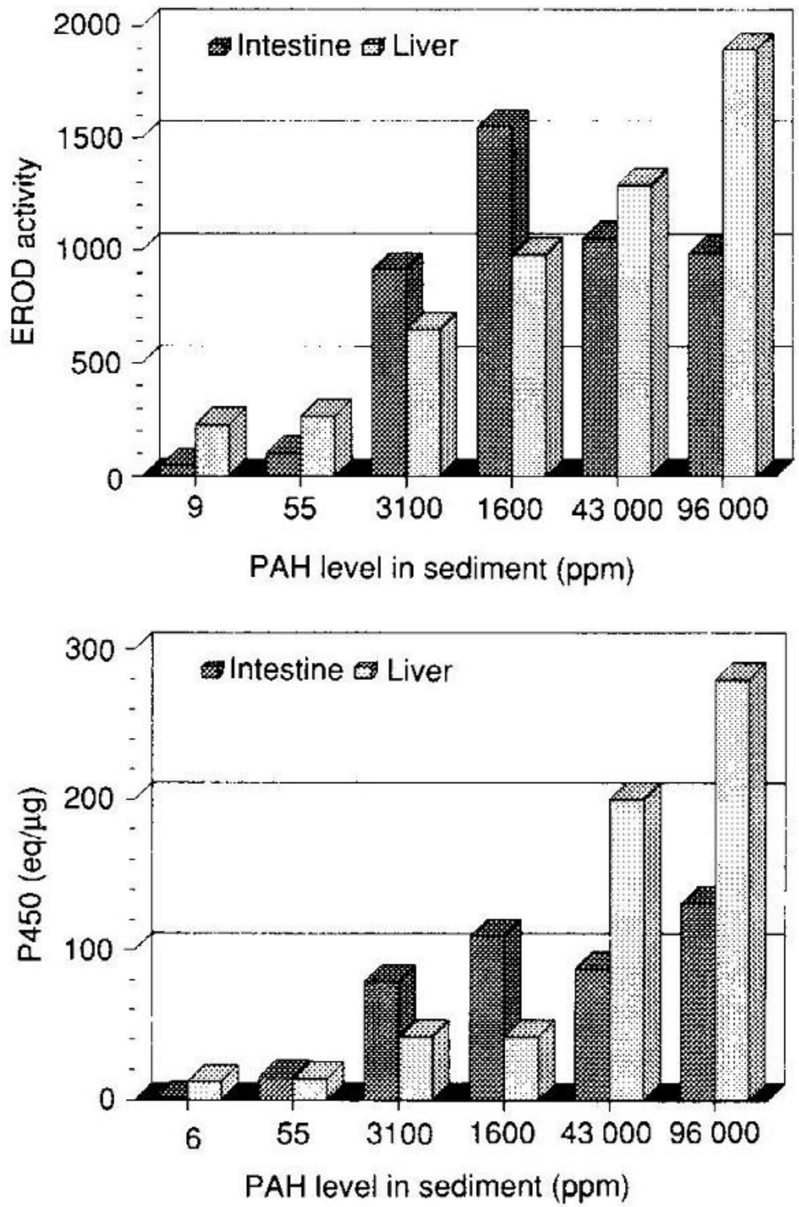


Figure 5.6 Relationship of sediment concentration of PAHs to EROD activity in liver and intestine of spot. After Van Veld *et al.* (1990).

Locality:

Reference Exposed

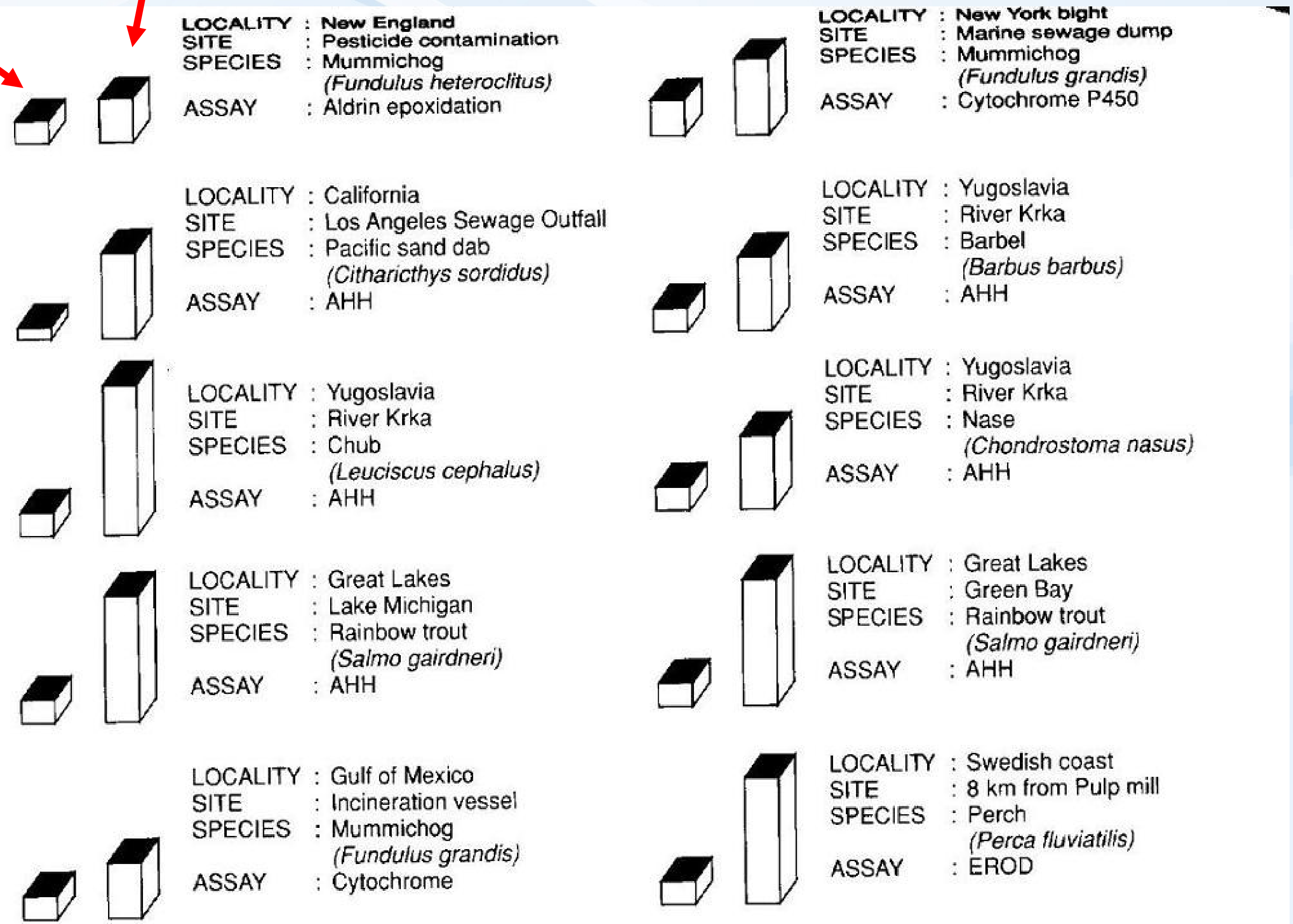
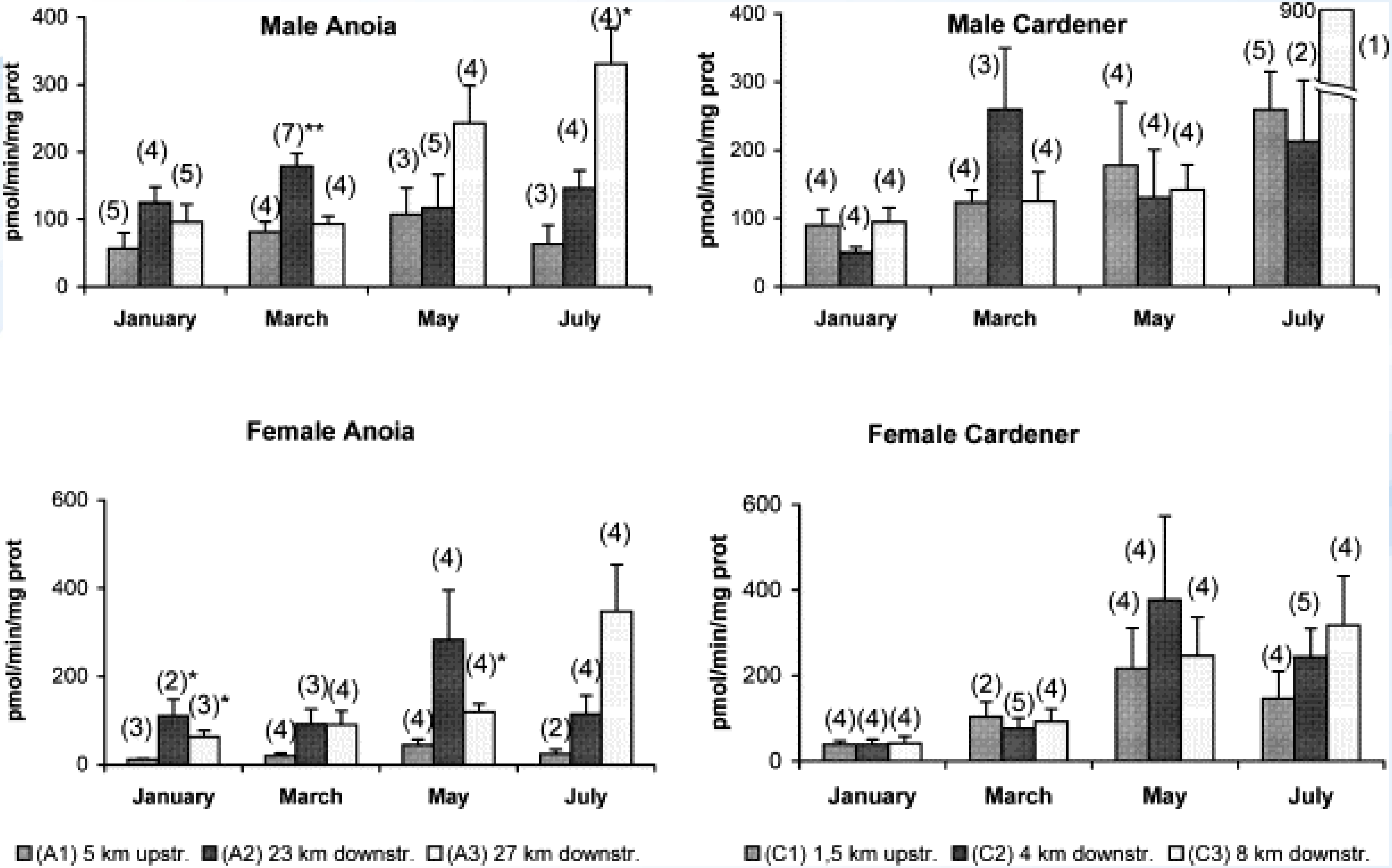


Figure 5.5 MFO changes in fish exposed to organic contamination. The proportion of either enzyme or cytochrome P450 levels detected at reference (short towers) and experimental sites (long towers) is presented in schematic form. All differences between reference and experimental sites were statistically significant ($P < 0.05$ or better). Payne *et al.* (1987).



EROD variation on male and female carp from the Anoia and Cardener tributaries
– *seasonal variability & response at contaminated localities*

MFO responses (EROD) are strongly **species specific** & not always related to clinical signs

Table 3.3 Comparison of the effects of PCB congeners on the reproduction of mink and rats

PCB congener	Mink	Rat
2,4,2′,4′-TCB	Clinically normal No change in cytochrome P450 No induction of MFO enzymes	Clinically normal No change in cytochrome P450 Some induction of MFO enzymes
3,3,3′,4′-TCB	Severe anorexia and diarrhoea Increase of cytochrome P450 No induction of MFO enzymes	Clinically normal Increase in cytochrome P450 Induction of MFO enzymes

After Gillette *et al.* (1987a).

MFO-responses (EROD) depends on animal size and **metabolism rate**

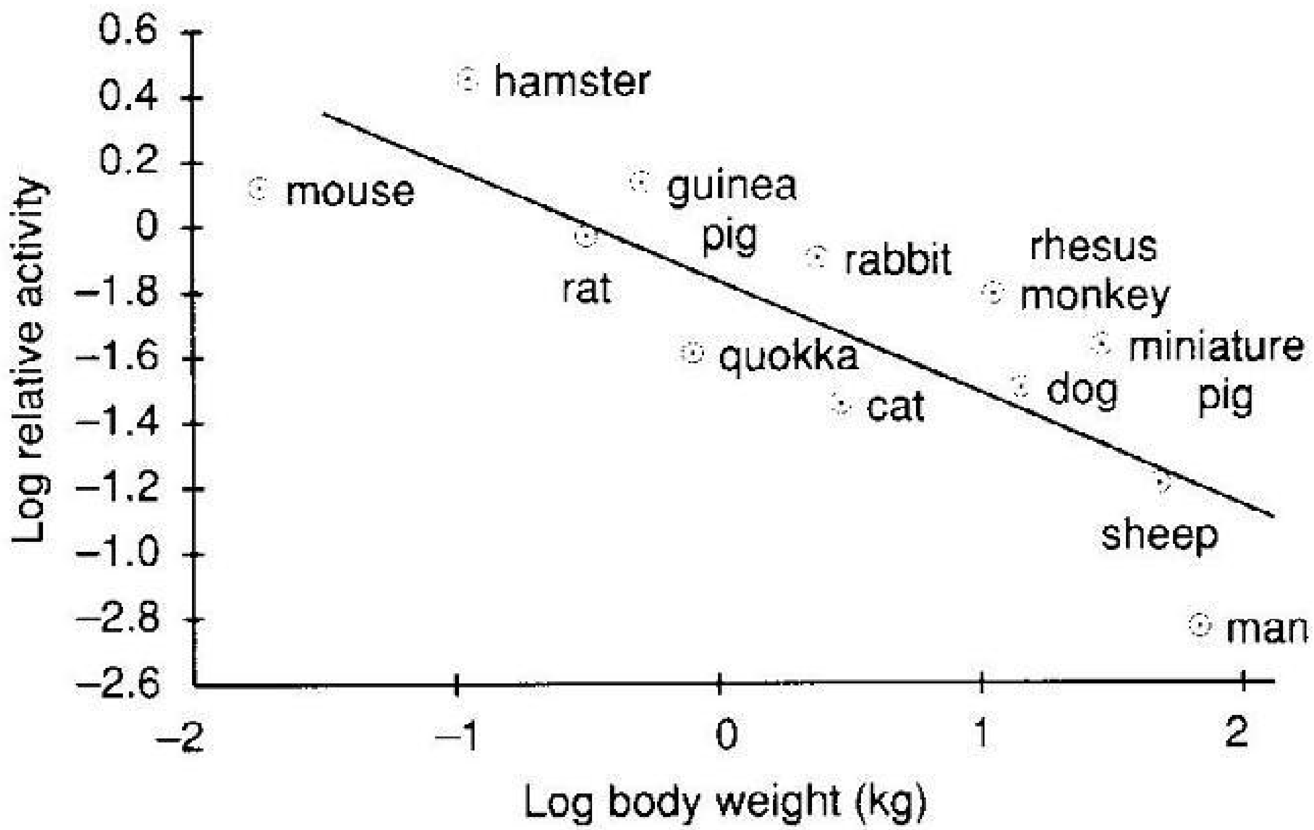


Figure 5.3 Relationship of body weight to MFO activity in mammals. Walker (1978 and 1980).

Centrum pro výzkum toxických látek v prostředí

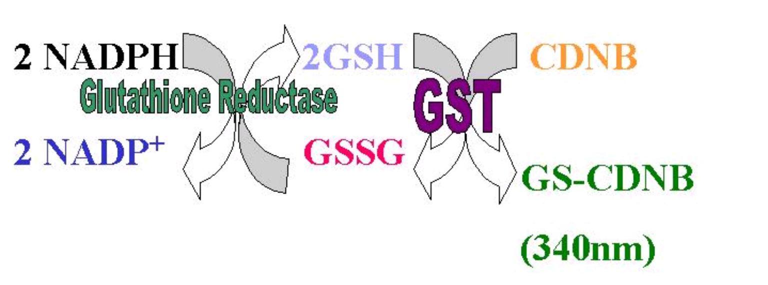
Phase II conjugation enzymes - GSTs

GSTs

soluble and membrane (endoplasmic reticulum) variants:
activities can be measured in cytoplasm or ER microsomes

Methods

Chemical reaction of
reduced GSH
+ thiol selective probe (CDNB)



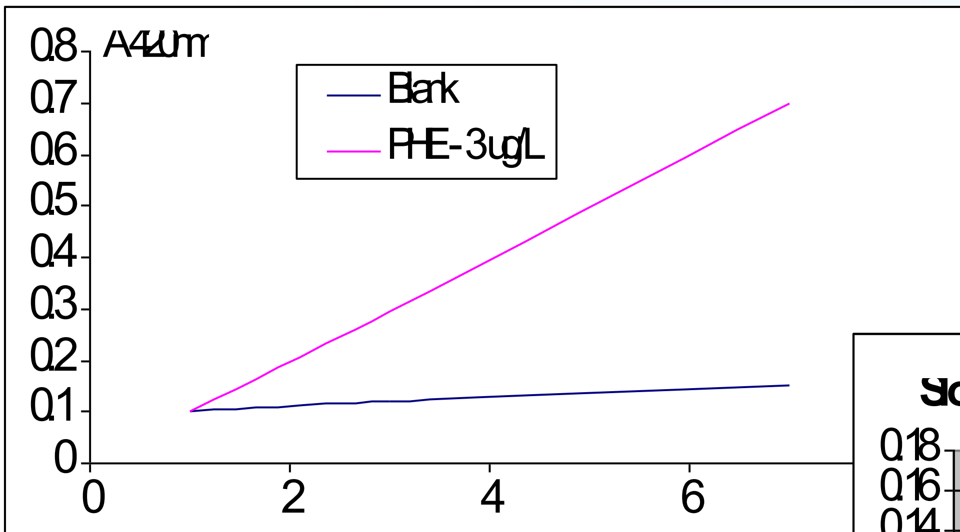
GST

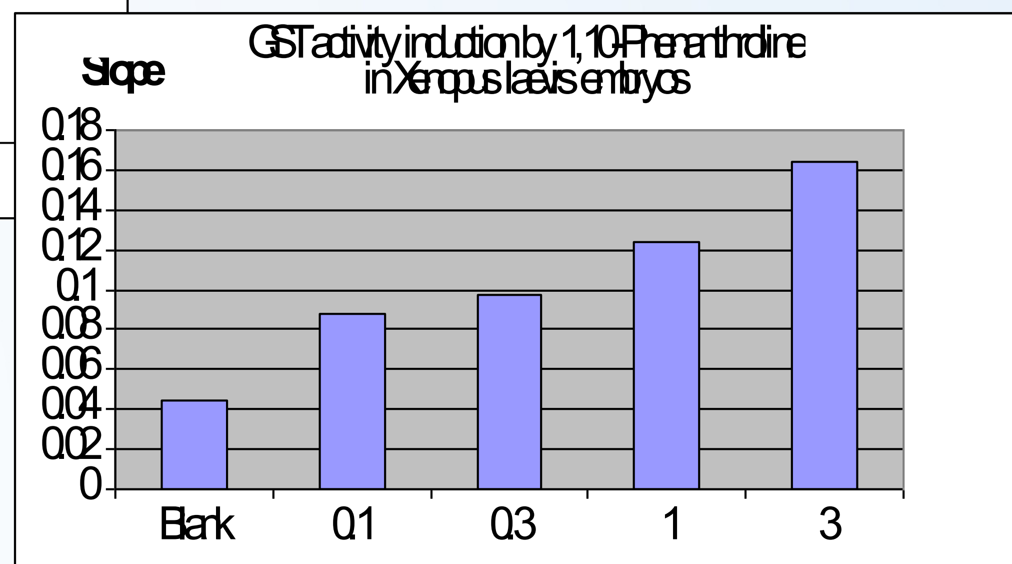
GSH + CDNB → **S-CDNB** (formation of coloured product)
kinetic or endpoint determination

GST activity determination: example

Kinetic assessment of GSTs

- stress → Induction of GSTs
- faster reaction = increasing slope of the kinetics





Biomarkers of oxidative stress

Centrum pro výzkum toxických látek v prostředí

Oxidative stress markers

Several parameters respond to oxidative stress

: **enzymes** – detoxification, antioxidants: GPx, GR, GSTs) ..
- enzymatic activities (see elsewhere)

: antioxidants – e.g. **GSH** (discussed further), vitamin E

: markers of oxidative damage
- membranes: **MDA** (discussed further)
- DNA: **8OH-dG**
(see at DNA damage / adducts-exposure biomarkers)
- proteins: oxidized forms (carbonyls)

Oxidative stress markers

GSH

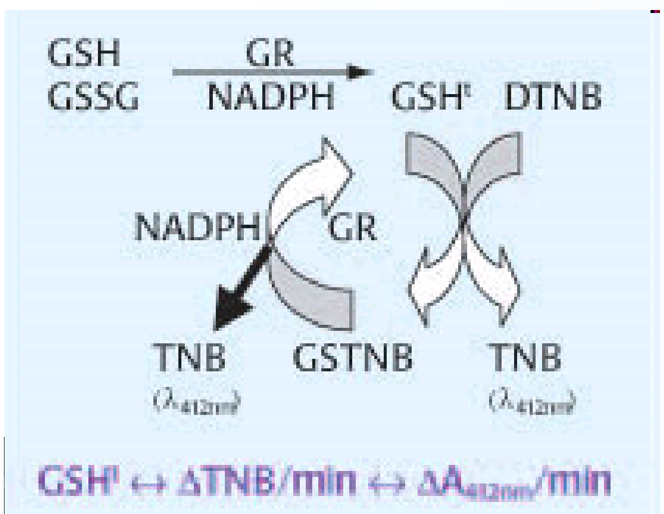
- antioxidant (scavenger of ROS) & reactive molecules
- conjugation molecules for detoxication
- probable intracellular regulatory molecule (? apoptosis ?)

Total glutathione = reduced GSH + oxidized GSSG

Method of determination (thiol selective probe DTNB)

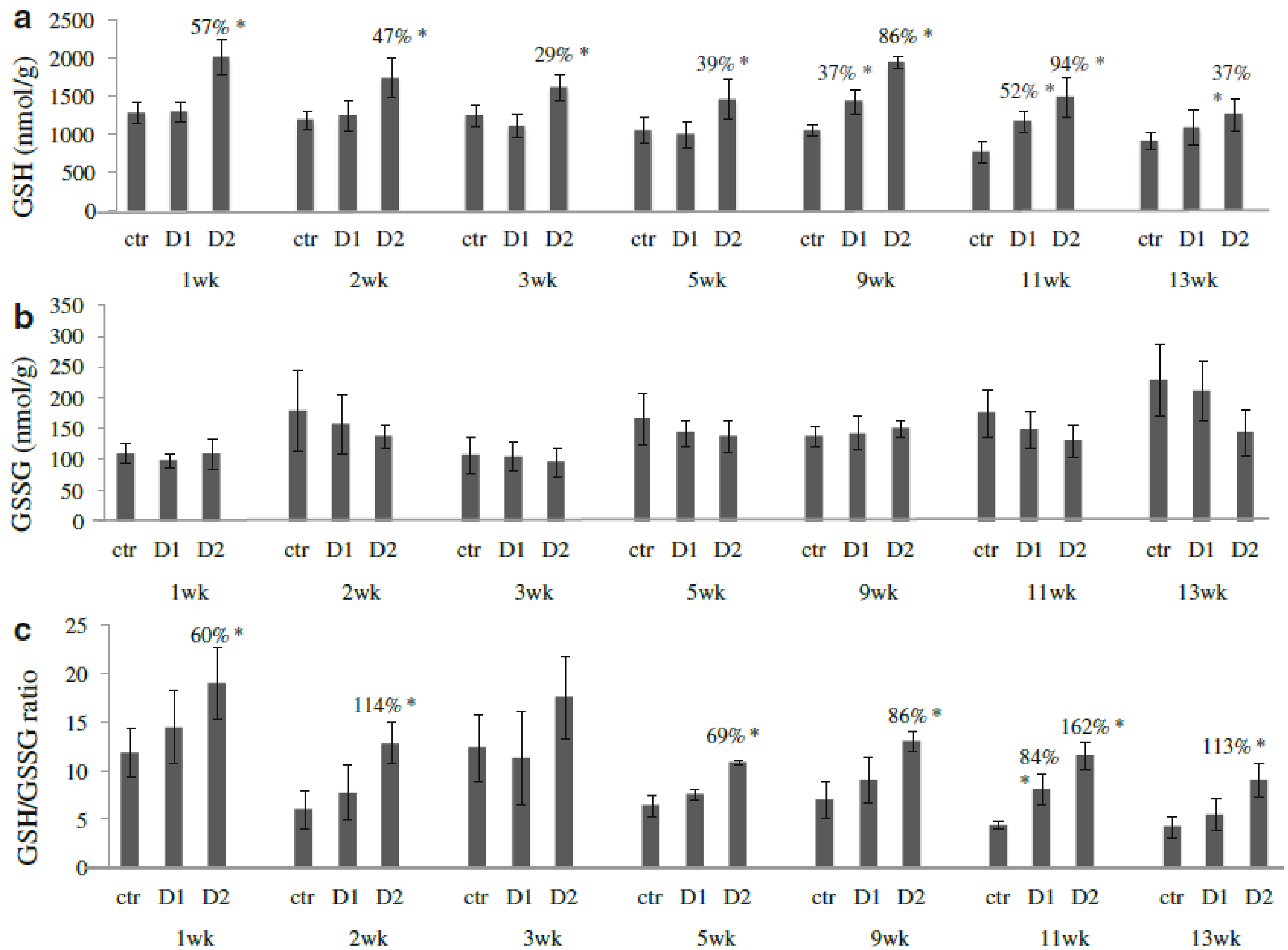
GSH + **Ellman s reagent (DTNB)** → Reduced GSH
GSH + GSH-reductase + **DTNB** → Total GSH

Total – Reduced = Oxidized



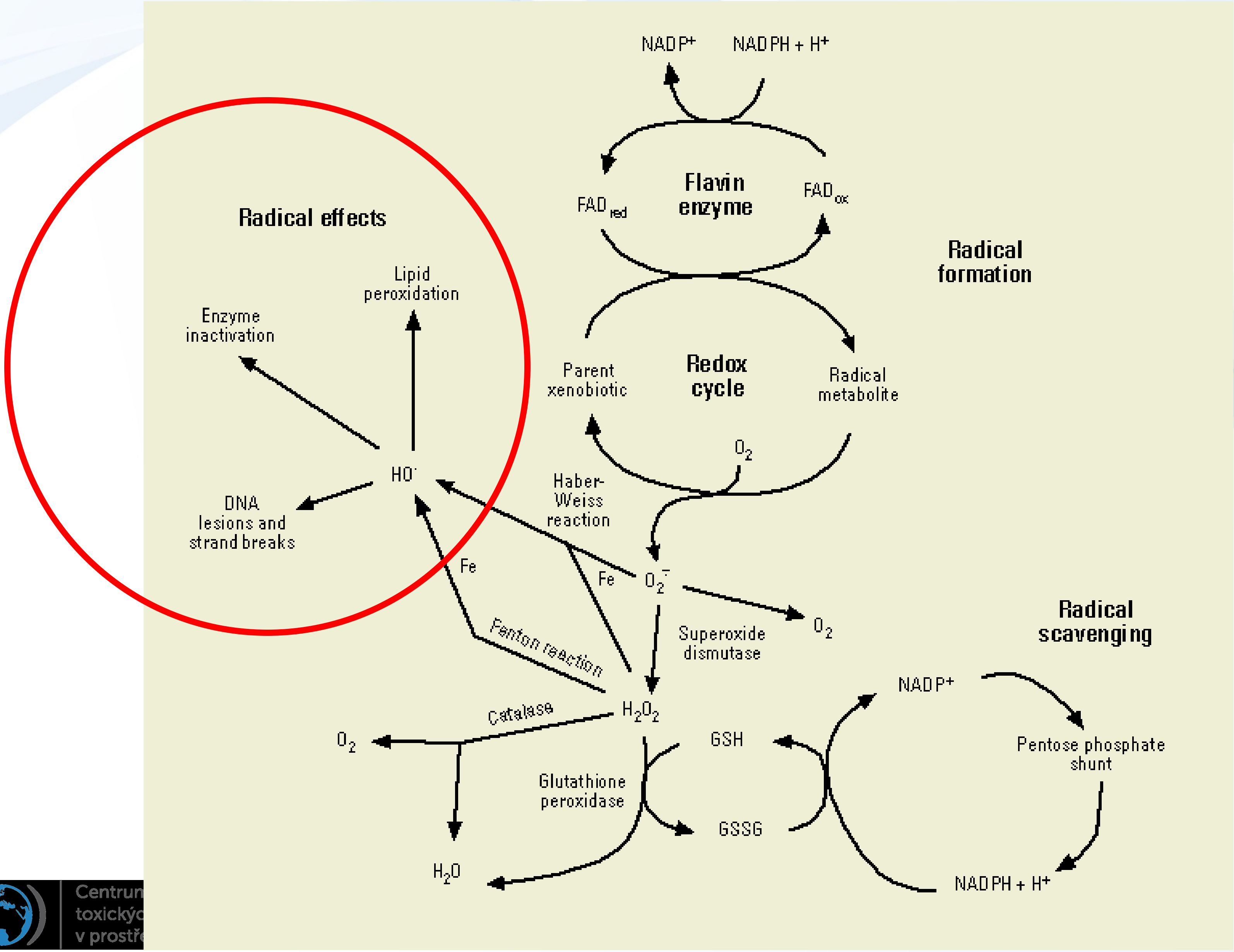
Example - GSH modulation by toxic nanoparticles

Fig. 6 Content of GSH (**a**), content of GSSG (**b**), and GSH/GSSG ratio (**c**) in lung of mice after chronic exposure (1–13 weeks) to CdO nanoparticles at dose 1 (*D1*) and dose 2 (*D2*). *Numbers with asterisk* (*) in the graph indicate significant differences compared to the control variant within the respective week ($p<0.05$; $N=5$ animals)



Bláhová et al. 2014 Anal Bional Chem 406:5867–5876

Markers of oxidative DAMAGE

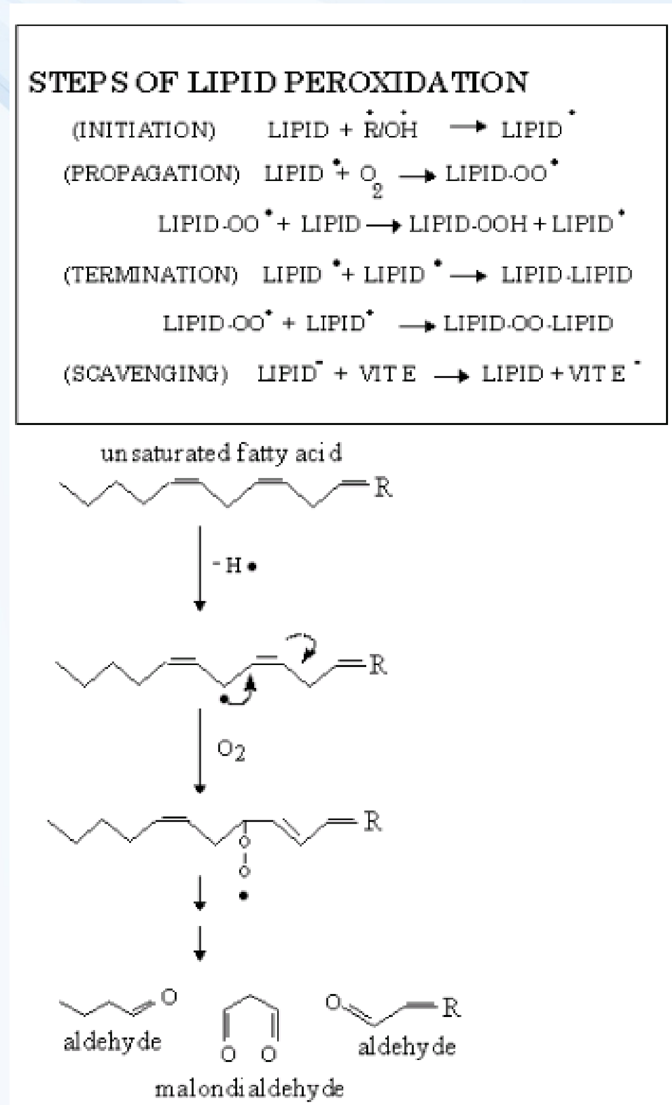


Centrum toxický v prostř

Lipid peroxidation → Malondialdehyde (MDA)

MDA – malondialdehyde

product of lipid peroxidation



Centrum pro výzkum toxických látek v prostředí

Malondialdehyde (MDA) determination

MDA – formed from oxidized membrane phospholipids

: determination:

- HPLC (instrumental)
- **TBARS (spectrophotometric) method**

TBARS – ThioBarbituric Acid Reactive Species

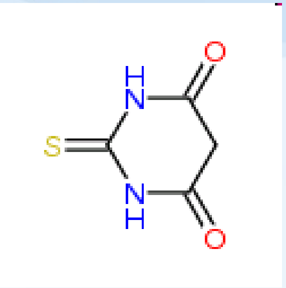
: less specific than HPLC

: easy determination (spectrophotometry)

Method:

1) sample extract *(with MDA)*
2) add TBA
3) boil (cca 30 / 90 C)

   → formation of **red/violet coloured product**
4) determination by spectrophotometry (A 540 nm)

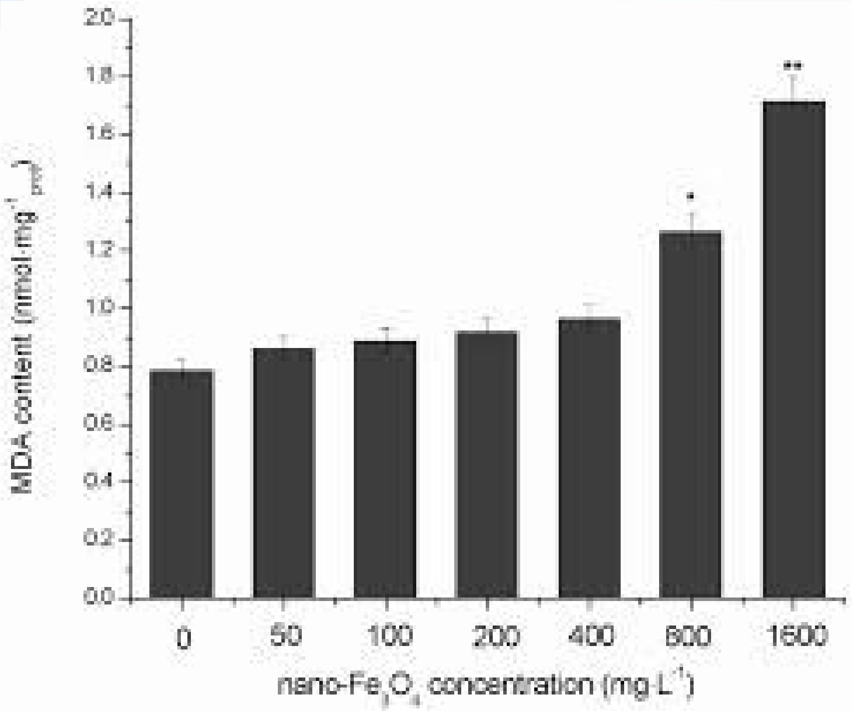


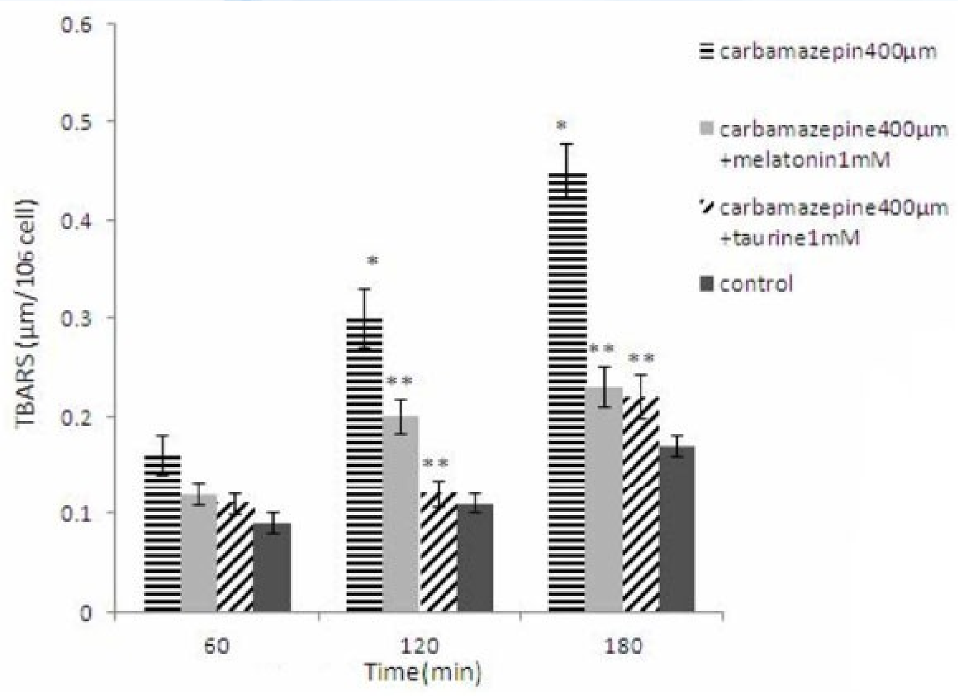
TBA

MDA modulation - examples

Effects of nanoFeOxide particles on MDA in fish

Induction of MDA (TBARS) by carbamazepine (and protection by antioxidants)





Centrum pro výzkum toxických látek v prostředí