Centrum pro výzkum toxických látek v prostředí

# BIOMARKERS AND TOXICITY MECHANISMS

# 12 – BIOMARKERS of EFFECTS

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www.recetox.cz

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

# In vivo biomarkers of effects / response

**Behavioral and Clinical biomarkers**

**Pathology**

**Clinical chemistry and hematology**

**Enzymatic changes**

**Protein synthesis biomarkers**

**Oxidative stress markers**

# Behavioral and clinical biomarkers

# 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**

# 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**

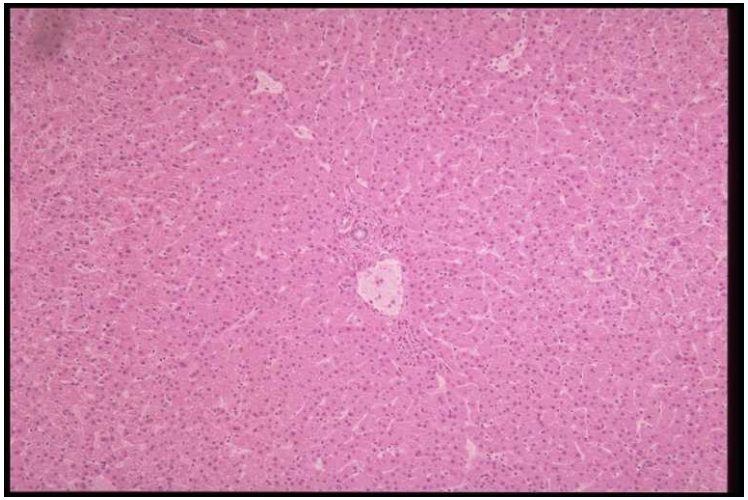
**(+) High relevance – organ/tissue changes**

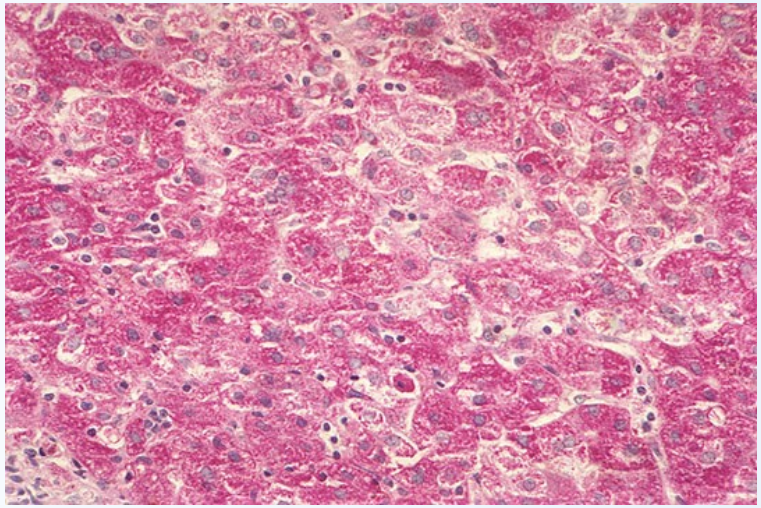
## 1) microscopy of internal organs

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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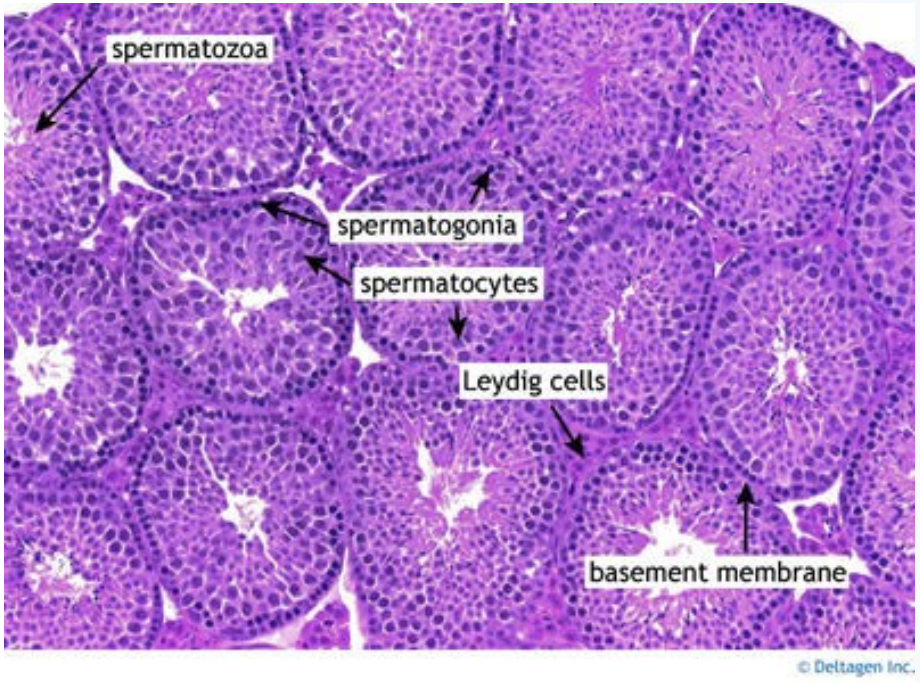
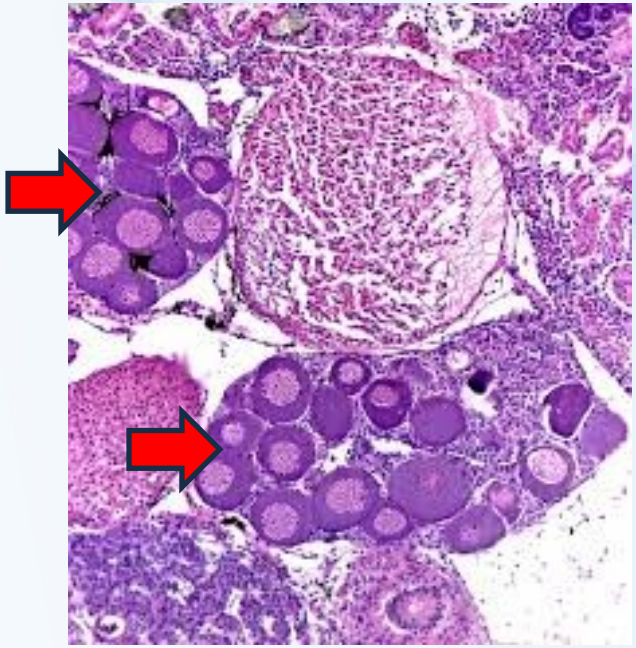
# Endocrine disruption: Intersex microscopy

## Testicular tissue




## Oocytes within testis



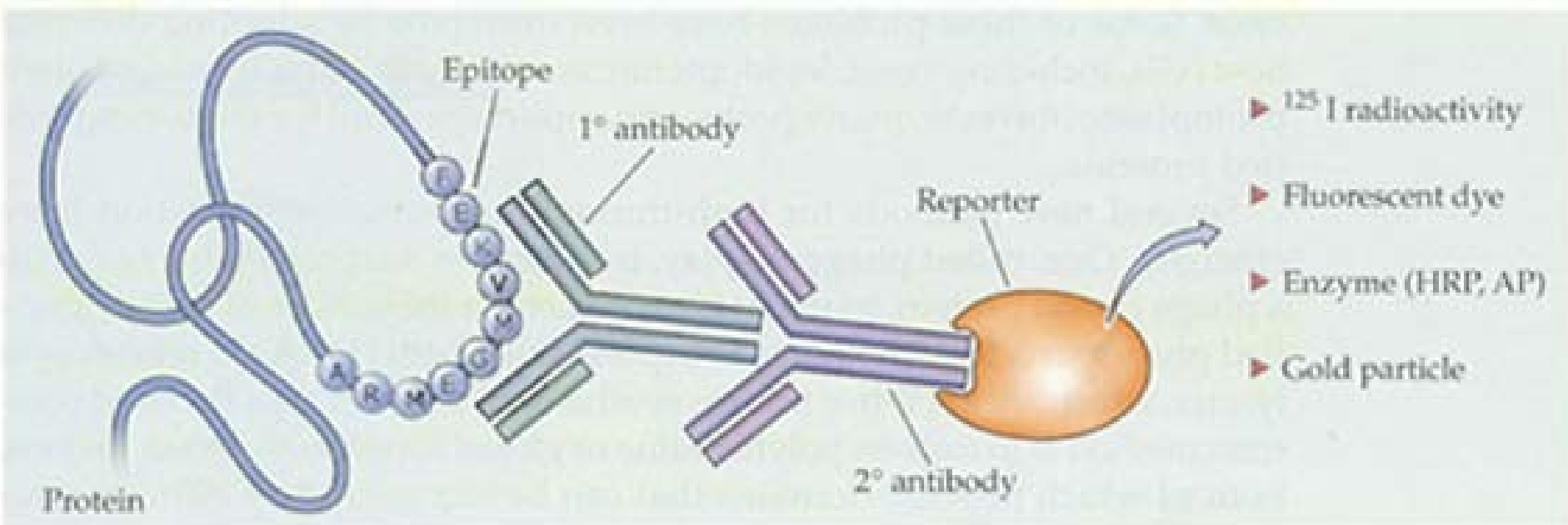
## 2) immunohistochemistry & microscopy

: determination of “specific” changes in tissues

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

Example → toxicant induced autoimmunity:

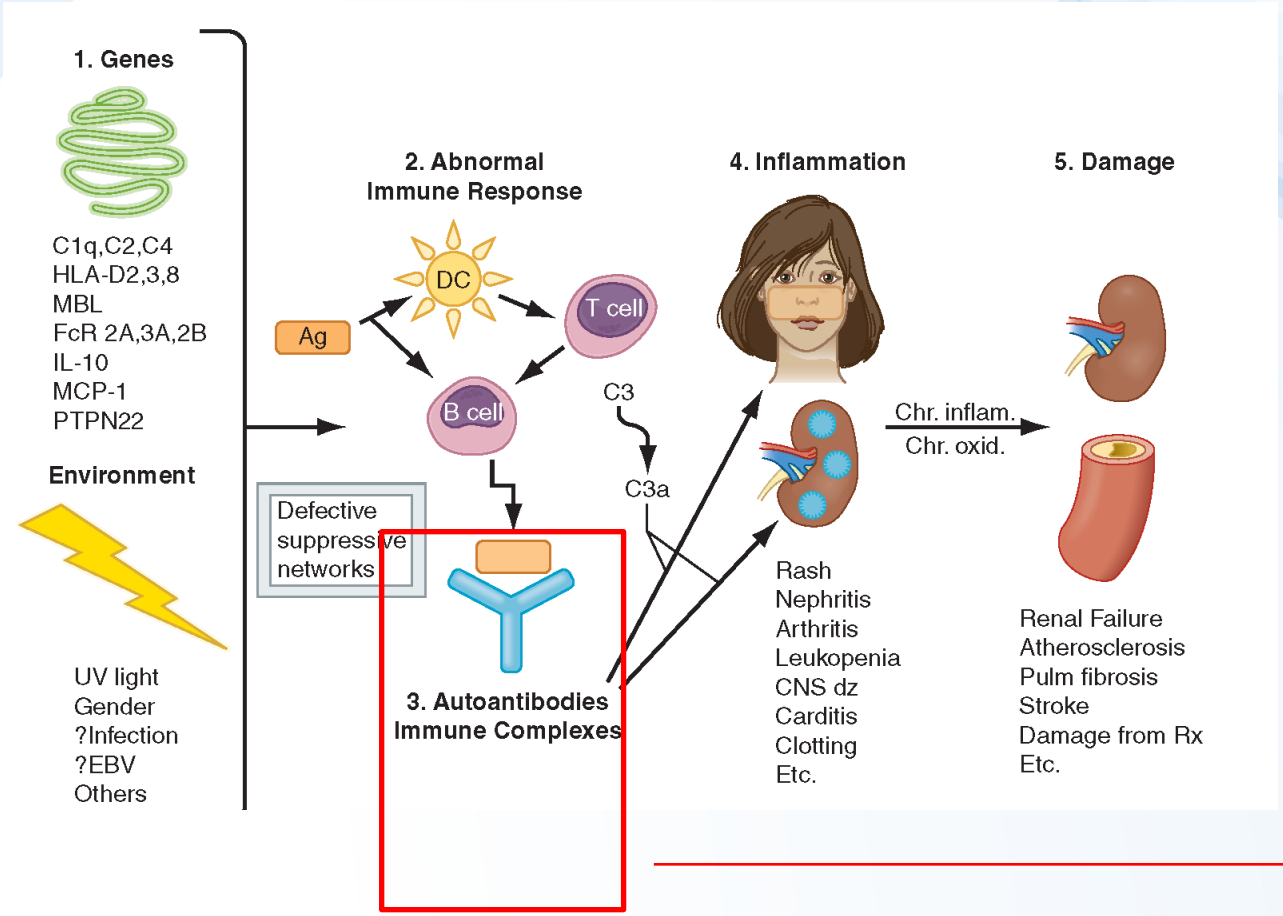
**anti-nuclear Ab (ANA test)**



# 2) immunohistochemistry & microscopy

## anti-nuclear Ab (ANA test)

Systemic lupus



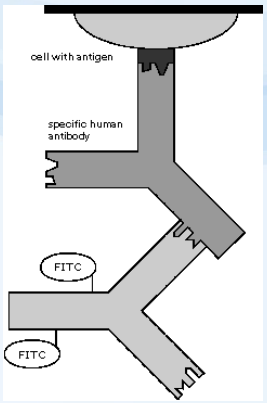

**ANA test**

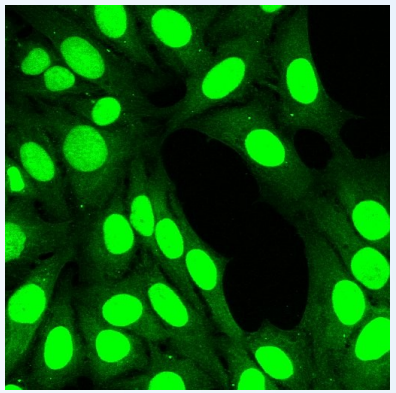
* Determination of antibodies in patient blood acting against “nuclei” proteins (ANA)

: target: permeated liver cells on slide

: application of blood (Ab)

: visualization (secondary Ab)



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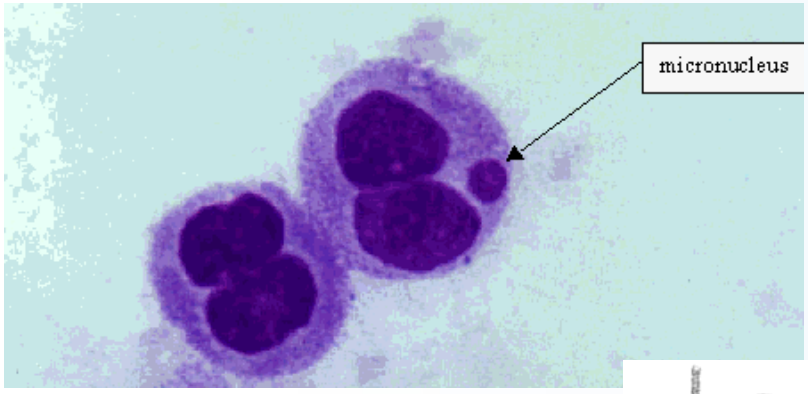
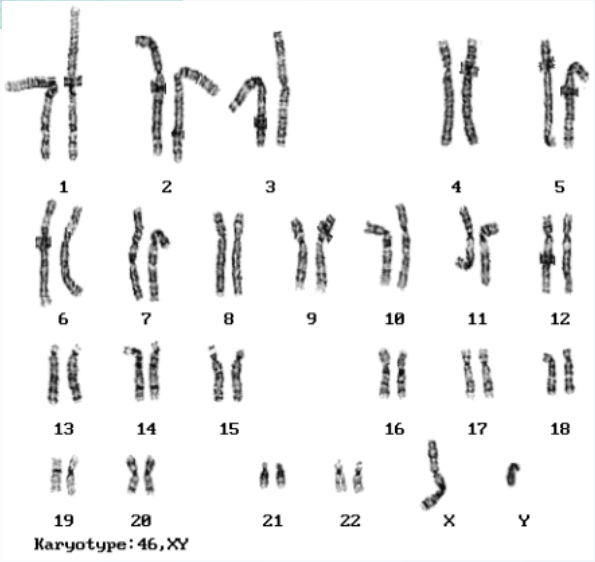
# Pathology

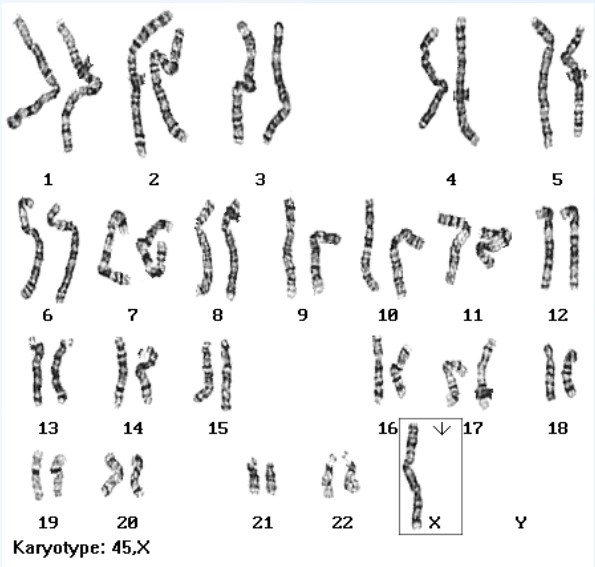
## 3) Nuclear DNA damage characterization

- **micronuclei evaluation by microscopy**
- **chromosomal abnormalities**

karyotype biomarkers (*human genetic disorders)*

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# Standard clinical chemistry & hematology biomarkers

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# 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.**

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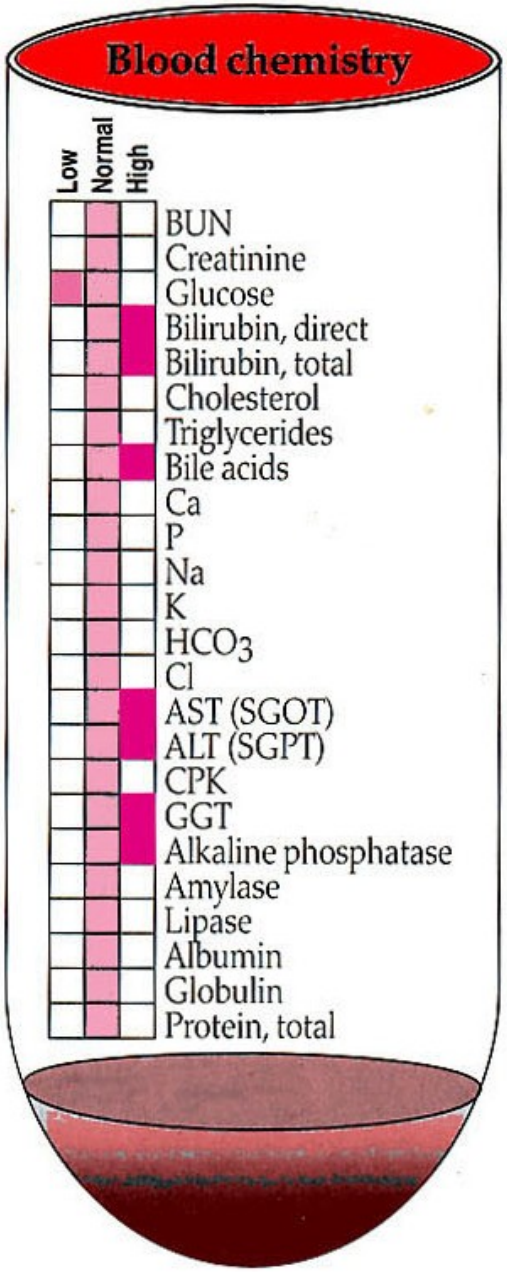
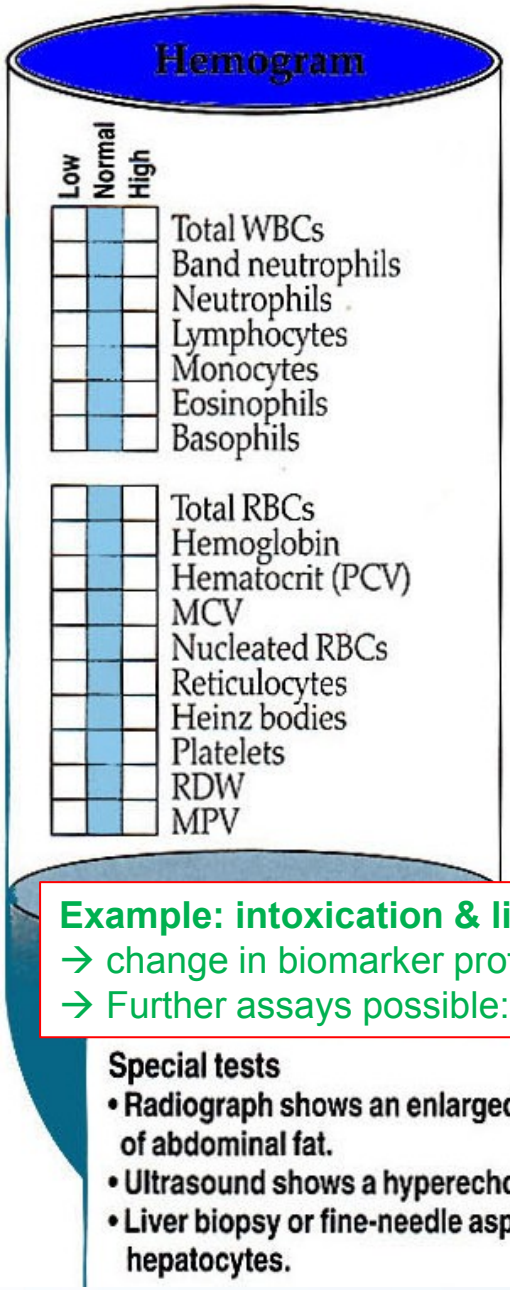
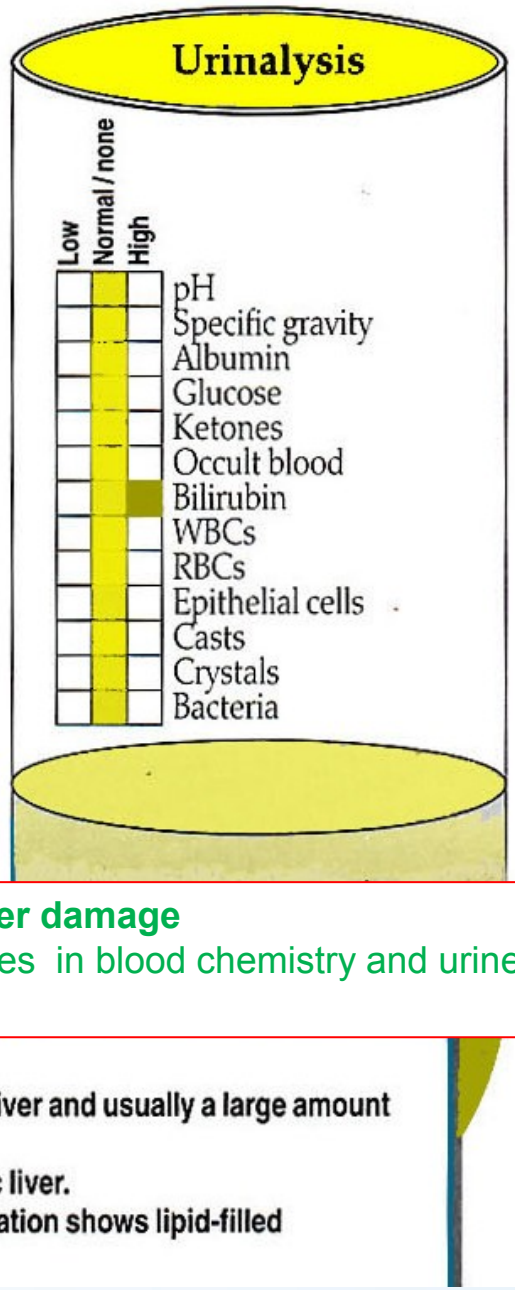



**Example: intoxication & liver damage**

→ 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);

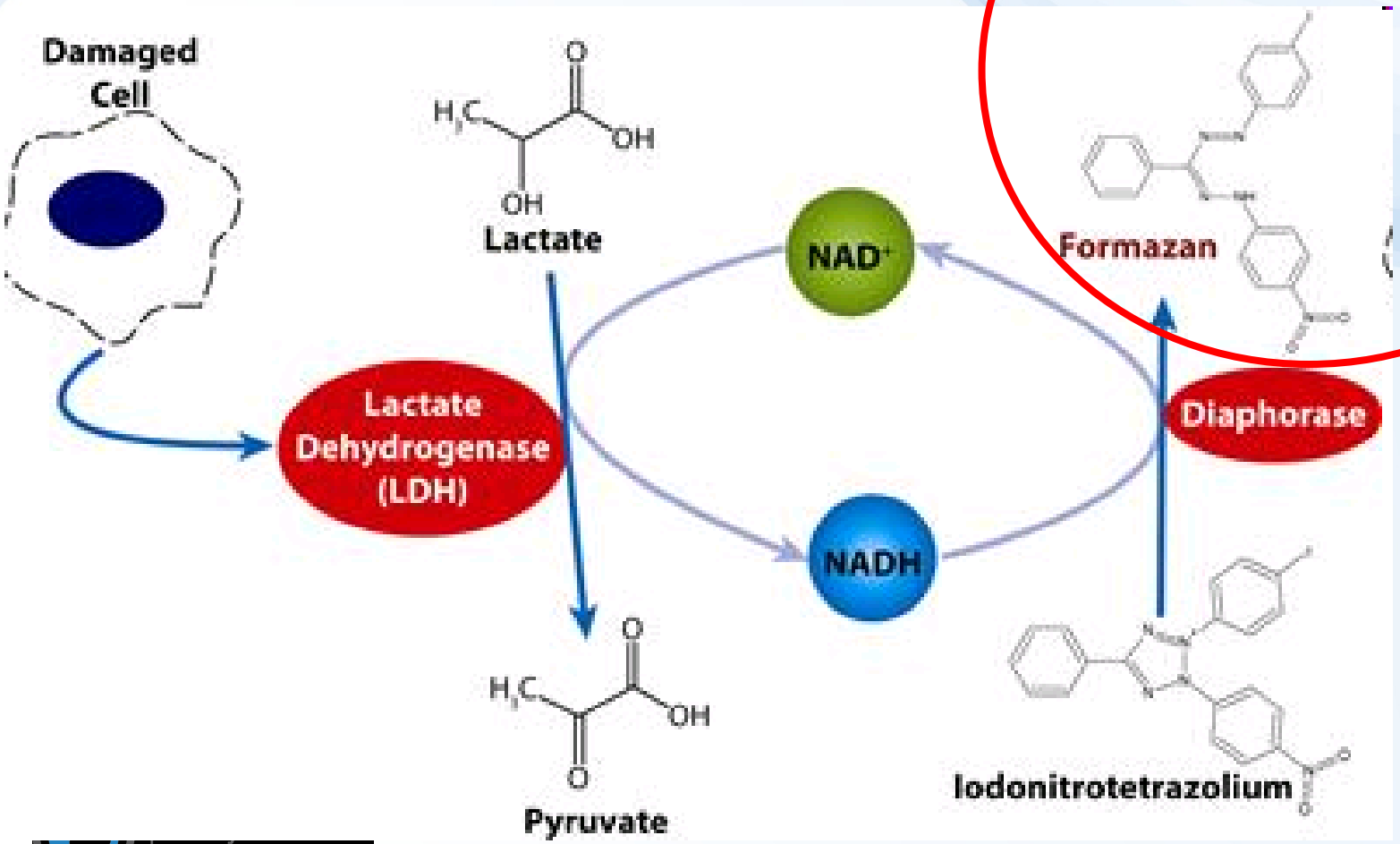
**Other enzyme biomarkers→ see further**

# 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

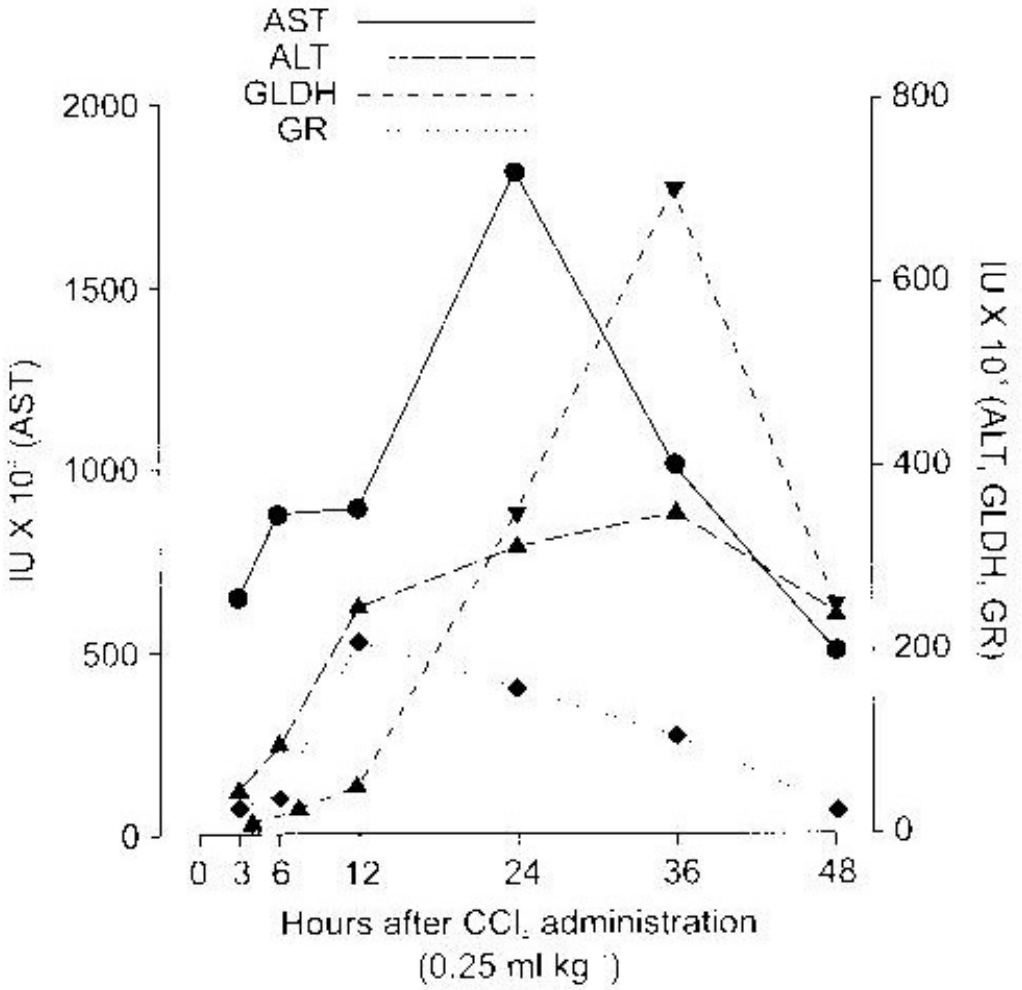



**Figure 3** Serum enzyme levels in rats following dosing with carbon tetrachloride ($CCl_4$, 0.25 ml $kg^{-1}$). Redrawn from Zimmerman (1978).

**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) |

# ESTABLISHED PROTEIN MARKERS – determination in blood

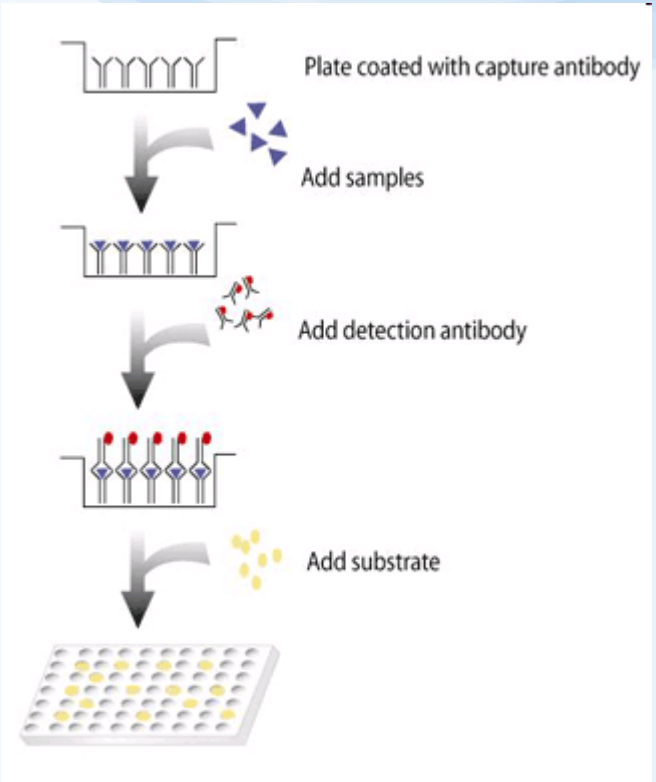
## Tumor genes and tumor markers

- cancer genes *ras, myc,*
- *α-fetoprotein (AFP)*
- suppressor genes *p53, Rb*

## Methods of determination in practice:

**ELISA**

(enzyme linked immunosorbent assays)



# Changes in enzyme activities

# Enzymatic changes

**Biomarkers reflecting „enzyme changes“:**

**Example biomarkers … inhibitions of**

AcChE (organo-phosphates)

d-Aminolevulinic Acid Dehydratase (ALAD) (lead - Pb)

Proteinphosphatases (microcystins)

**Inductions of detoxication & oxidative stress enzymes**

(hepatopancreas / liver / blood)

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

**Phase II enzymes** (GSTs)

Glutathion metabolism enzymes (GPx, GRs)

(+) Rapid enzymatic assays, specific responses

(-) Some ~ EXPOSURE biomarkers

# Reminder: AcChE inhibition mechanism

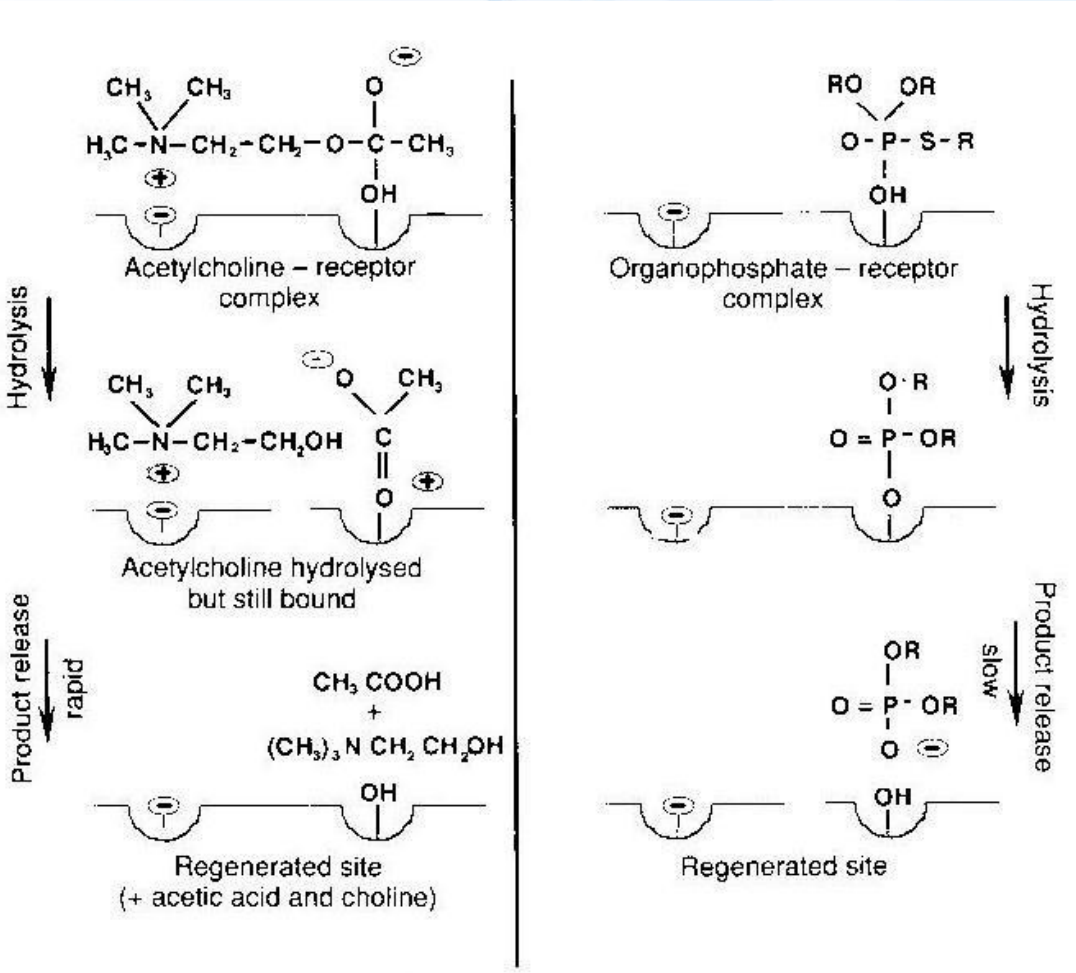


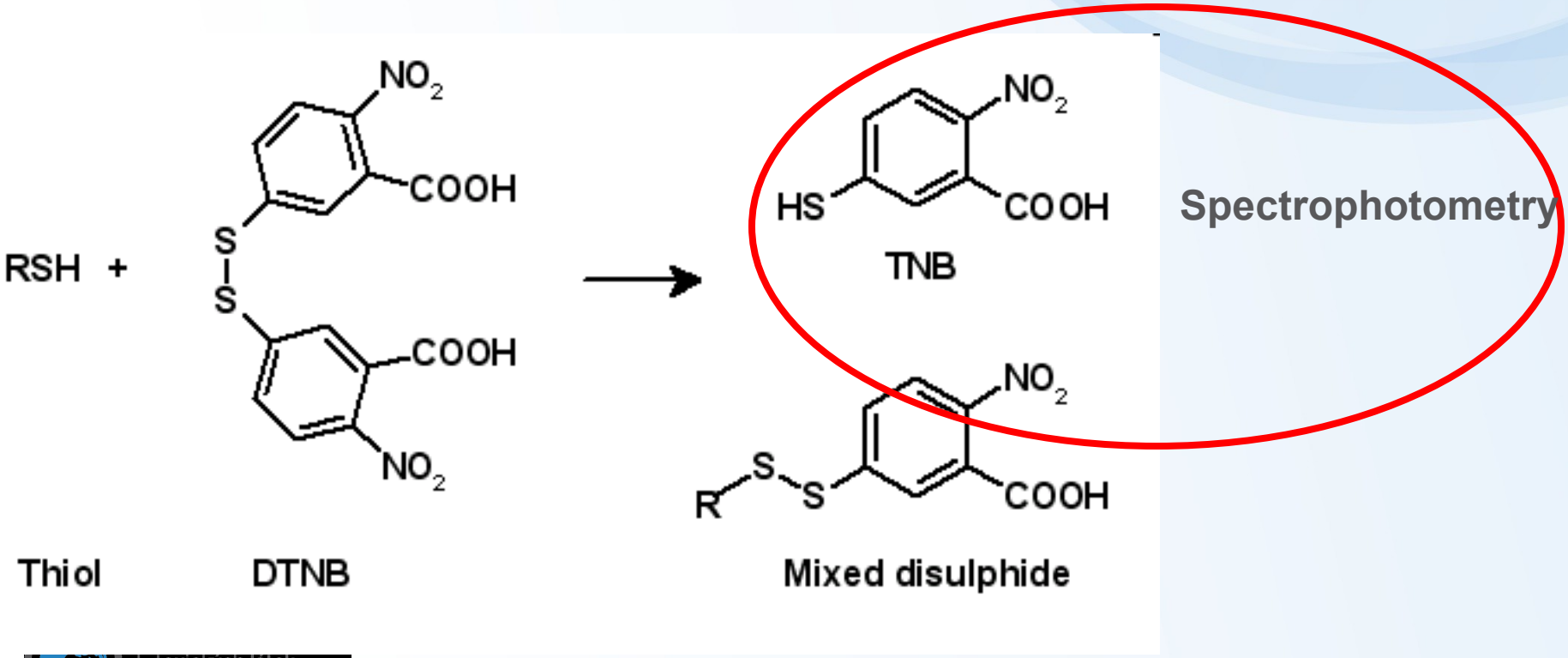

**Figure 2.2** Mode of action of inhibition of acetylcholinesterase.

# 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

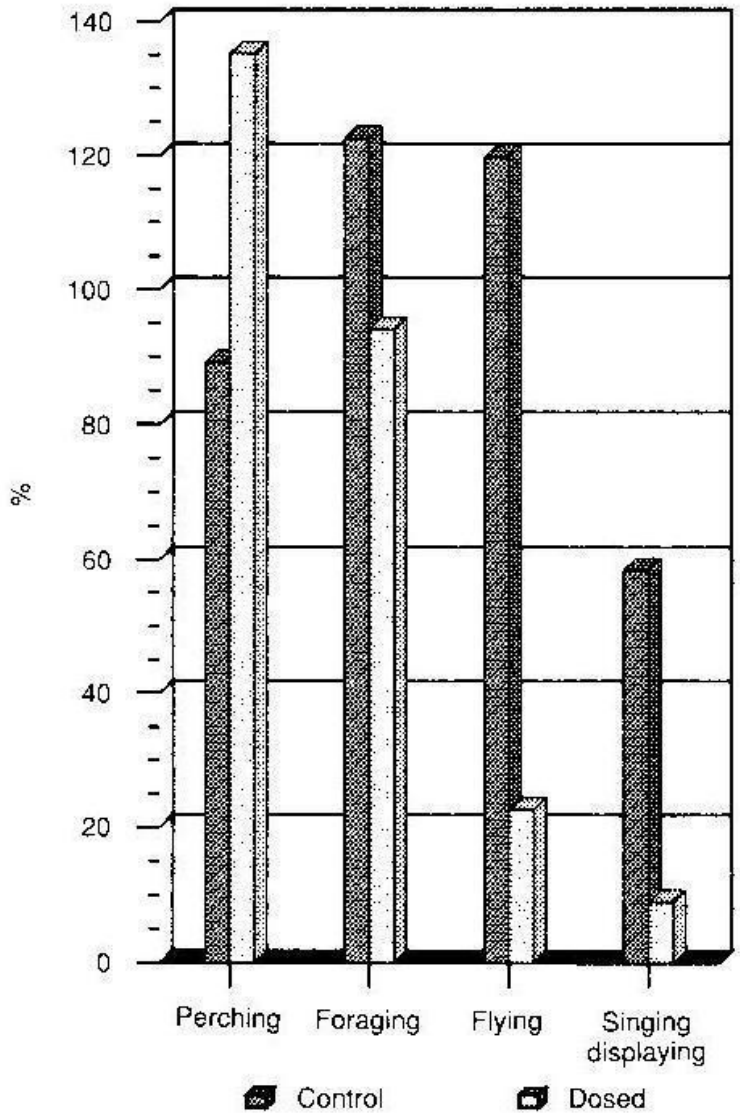



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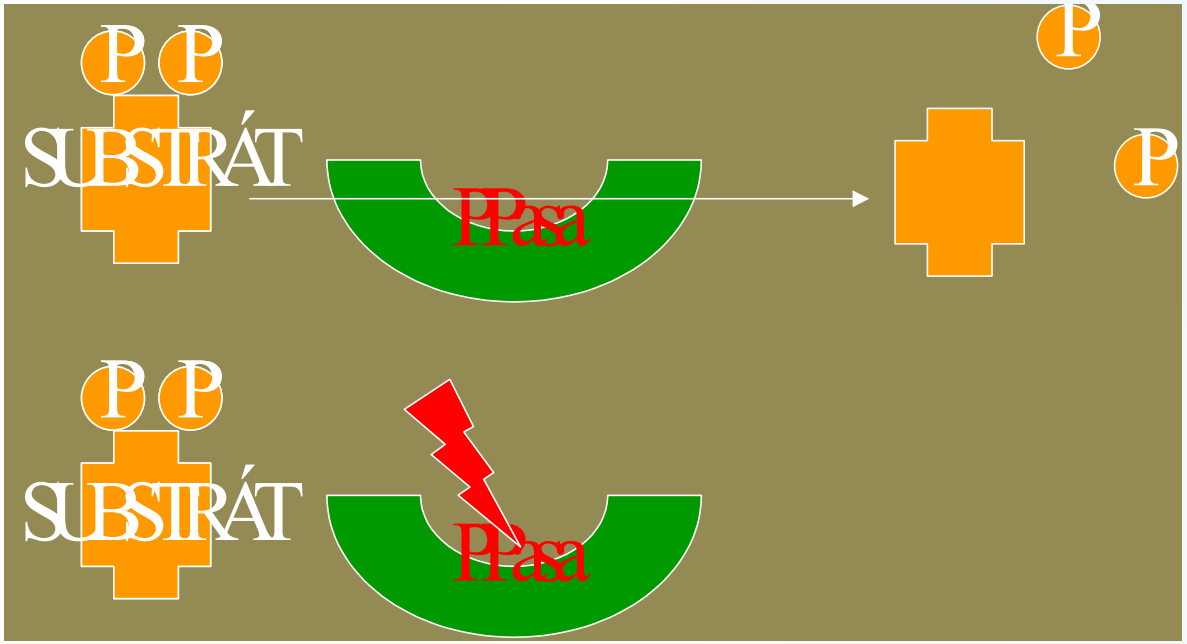
**Figure 2.4** Effect of OP on behaviour of starlings. After Grue and Shipley (1981).

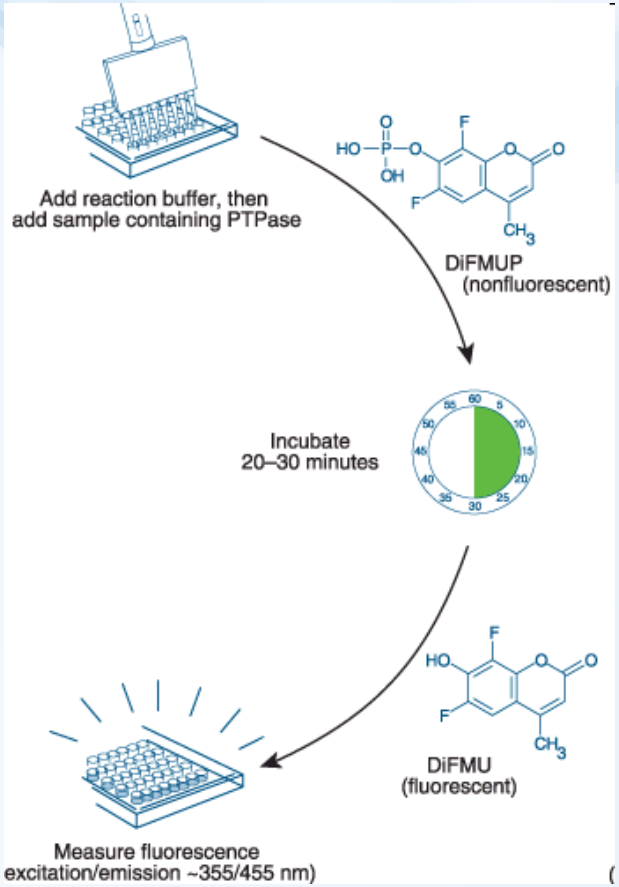
# Proteinphosphatase inhibition assay

## **Model substrates** cleaved by PPase

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

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

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# MFO (CYPs) - reminder

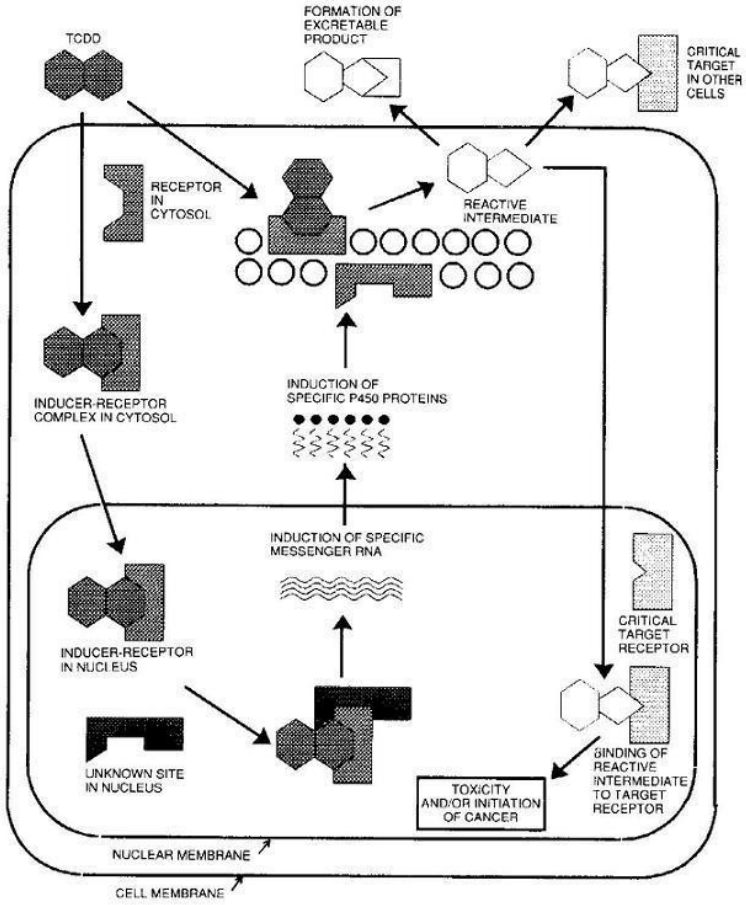



**Figure 5.1** Diagram of MFO system. Nebert and Gonzalez (1987).

**Table 5.1** Classification of P450s

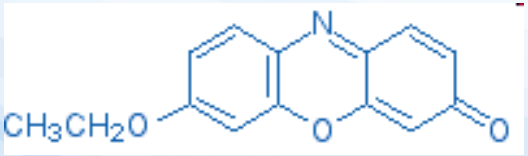
| Nomenclature | Induced by/specificity |
|---|---|
| P450I | Polycyclic aromatic, TCDD |
| P450II | Phenobarbital-inducible family* |
| P450IIA | Specific for testosterone hydroxylase |
| P450IIB | PB inducible |
| P450IIC | PB inducible |
| P450IID | Specific for debrisoquine 4-hydroxylase |
| P450IIE | 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 – "EROD"

Determination of CYP1A1 activity

**"EROD" -** EthoxyResorufin-O-Deethylase activity




Substrate: **Ethoxyresorufin**

: Oxidation by CYP1A1

→ Fluorescence (easy determination)

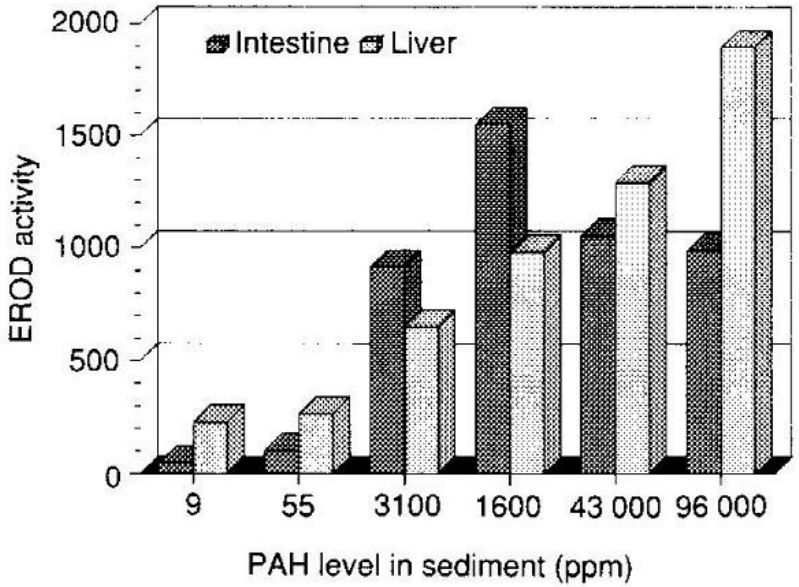
**EROD = sensitive biomarker of organic pollution (exposure & effects)**

: 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 …*



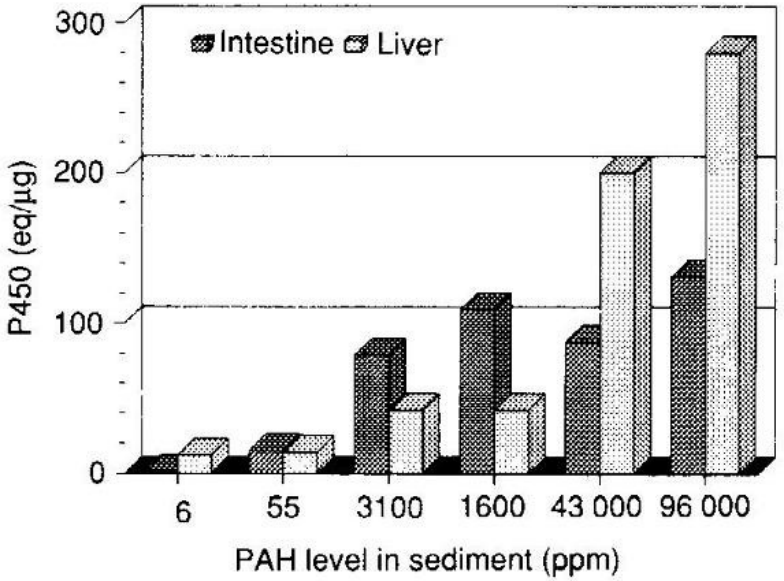




**Figure 5.6** Relationship of sediment concentration of PAHs to EROD activity in liver and intestine of spot. After Van Veld *et al.* (1990).

Locality:
Reference Exposed

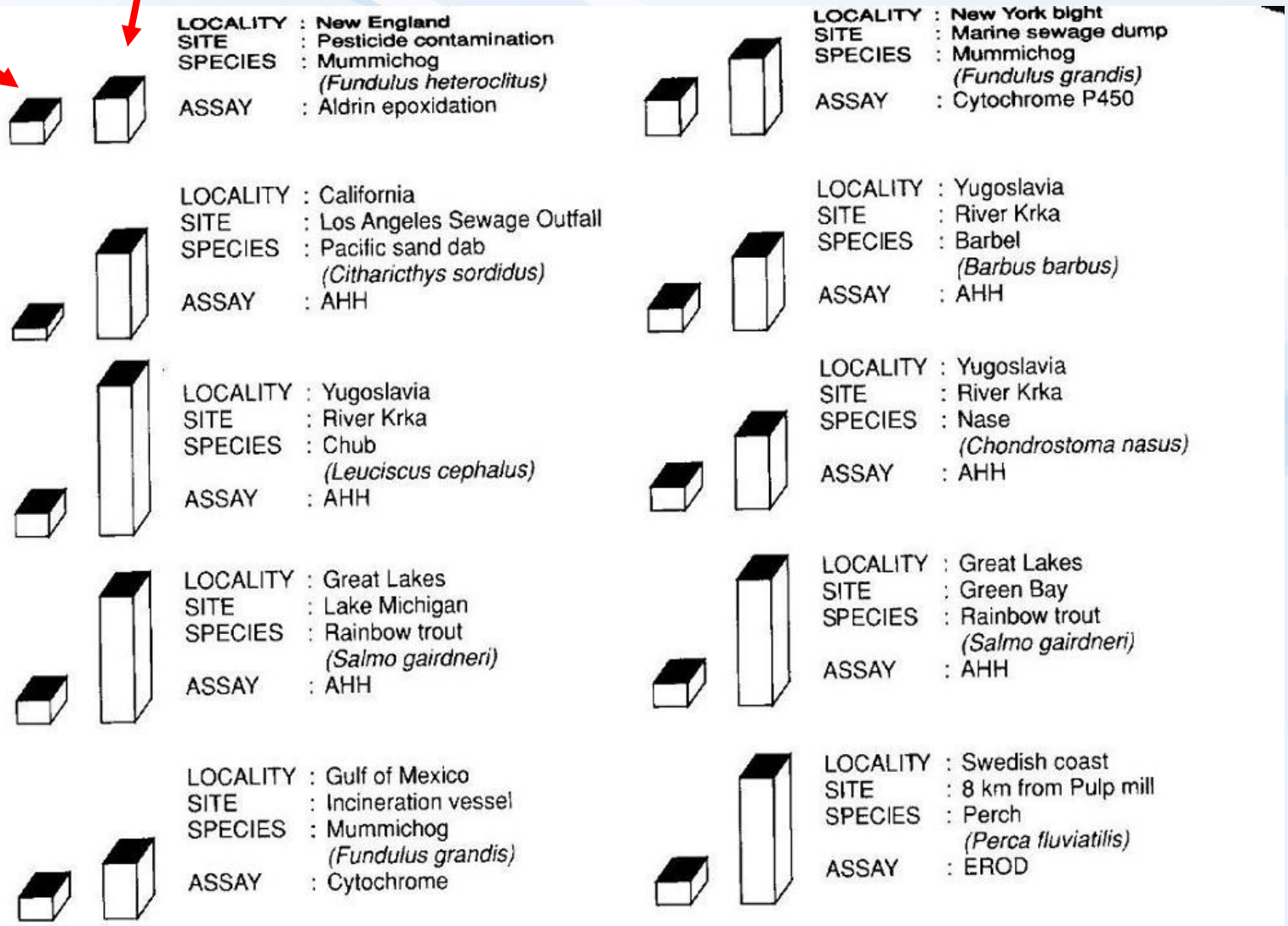
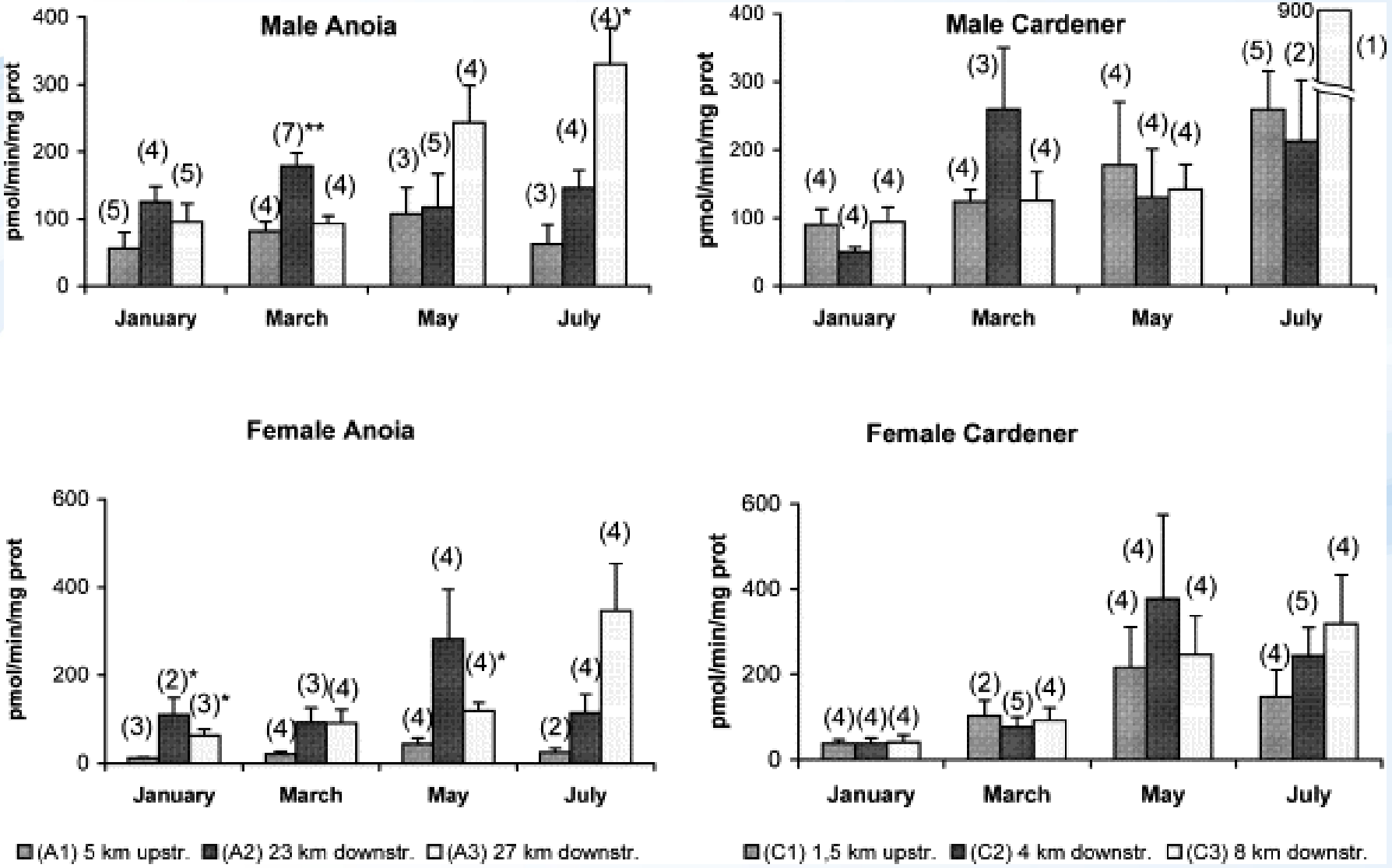



**Figure 5.5** MFO changes in fish exposed to organic contamination. The proportion of either enzyme or cytochrome P450 levels detected at reference (short towers) and experimental sites (long towers) is presented in schematic form. All differences between reference and experimental sites were statistically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

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# MFO responses 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<br>No change in cytochrome P450<br>No induction of MFO enzymes | Clinically normal<br>No change in cytochrome P450<br>Some induction of MFO enzymes |
| 3,3,3′,4′-TCB | Severe anorexia and diarrhoea<br>Increase of cytochrome P450<br>No induction of MFO enzymes | Clinically normal<br>Increase in cytochrome P450<br>Induction of MFO enzymes |

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

# MFO-responses depends on animal size and metabolism rate

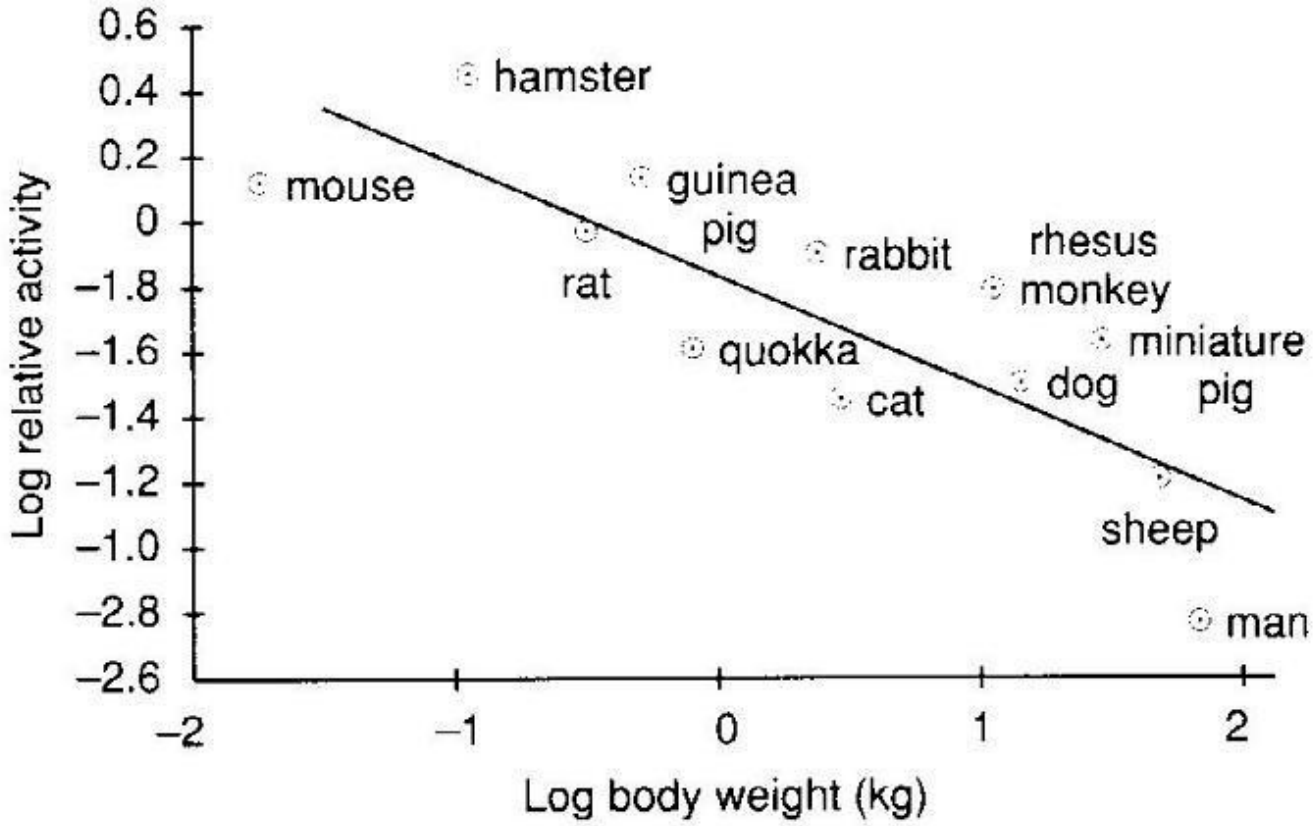



**Figure 5.3** Relationship of body weight to MFO activity in mammals. Walker (1978 and 1980).

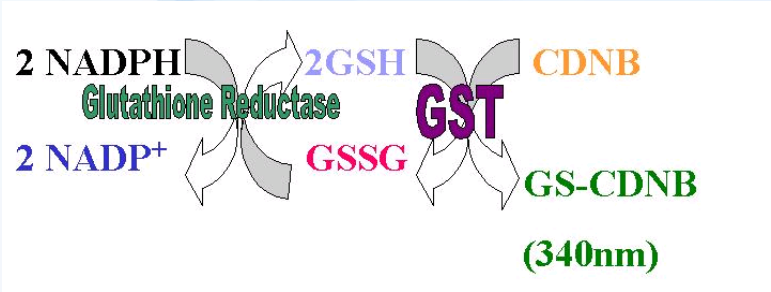
# Phase II conjugation enzymes - GSTs

**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 → **GS-CDNB** (formation of yellow product)
*kinetic or endpoint determination*

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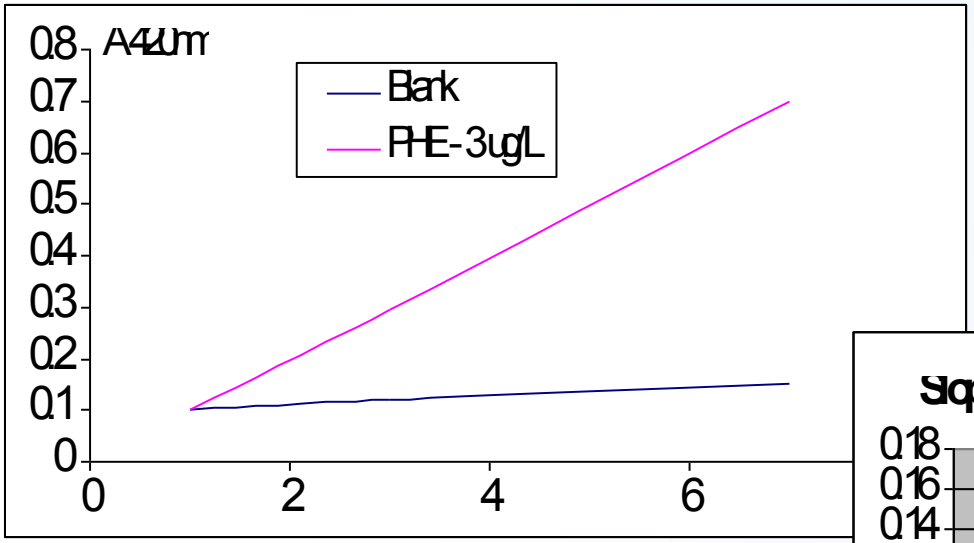
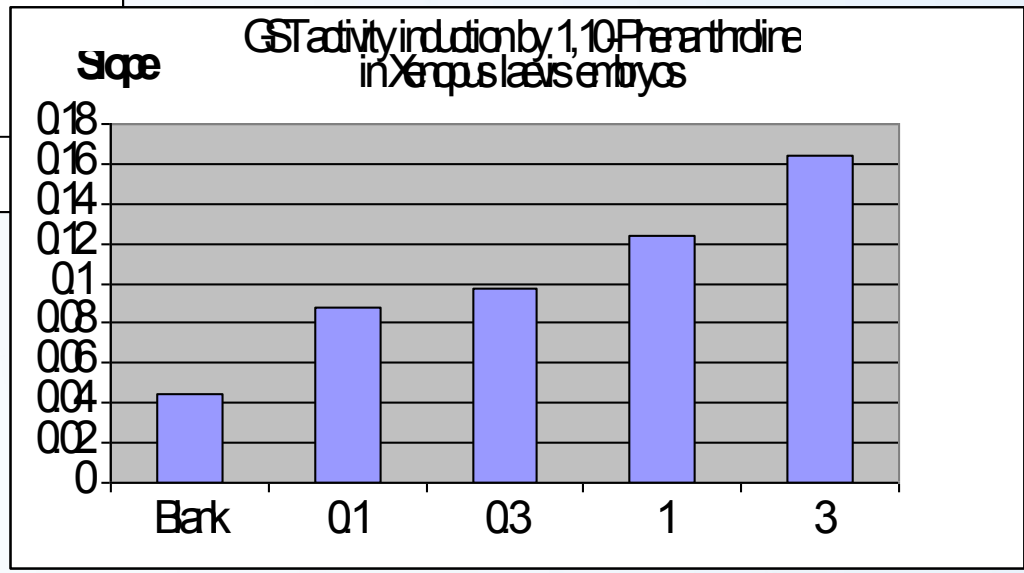
# GST activity determination: example

Kinetic assessment of GSTs

stress → Induction of GSTs

faster reaction = increasing slope of the kinetics

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# Biomarkers - protein expression

# PROTEIN SYNTHESIS

## Protein determination

- amount (concentration)
- activity (see enzymatic assays)

## Amount quantification

- mRNA levels
- protein levels
  - electrophoresis and Western-(immuno)blotting
  - ELISA techniques

## Examples of protein biomarkers

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

metalothioneins

Vitellogenin(-like) Vtg proteins in male

Aromatase

# Heat Shock Proteins (hsp)

## General stress = synthesis of new proteins

~ equilibrium and homeostasis buffering

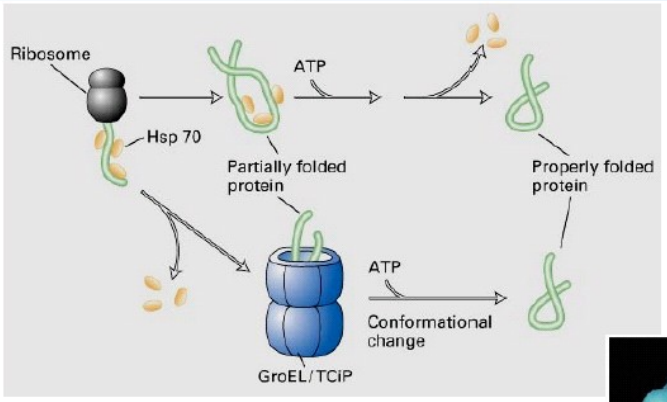
- temperature (cold / heat) → proteins assuring cryo-preservation
- salinity & metals → ion buffering
- organic xenobiotics → detoxication

**New proteins must be folded to their 3D stucture**

by activity of **„CHAPERONES“**

Chaperons = hsp90, hsp60, hsp 70

*~ 60-90 kD molecular weight kD*



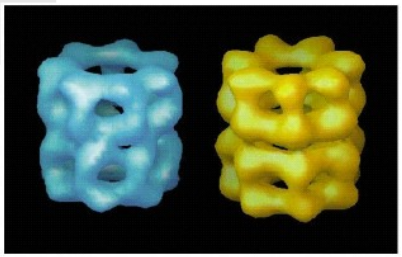
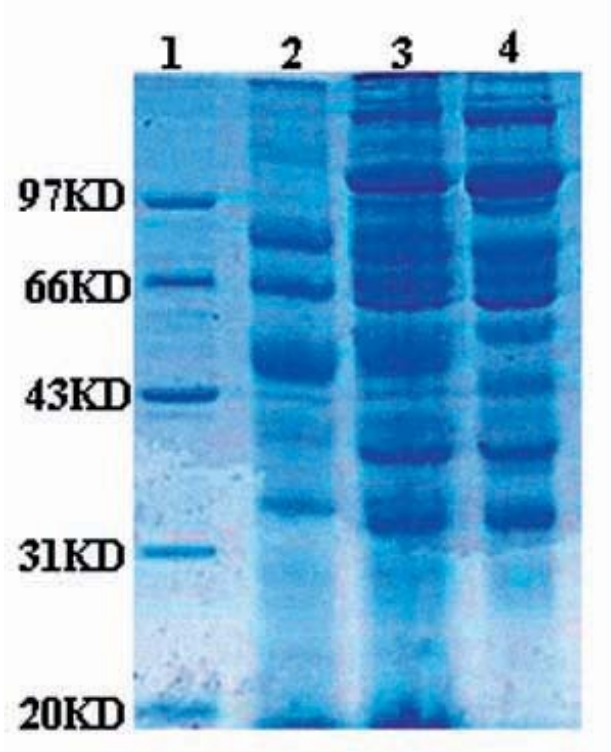



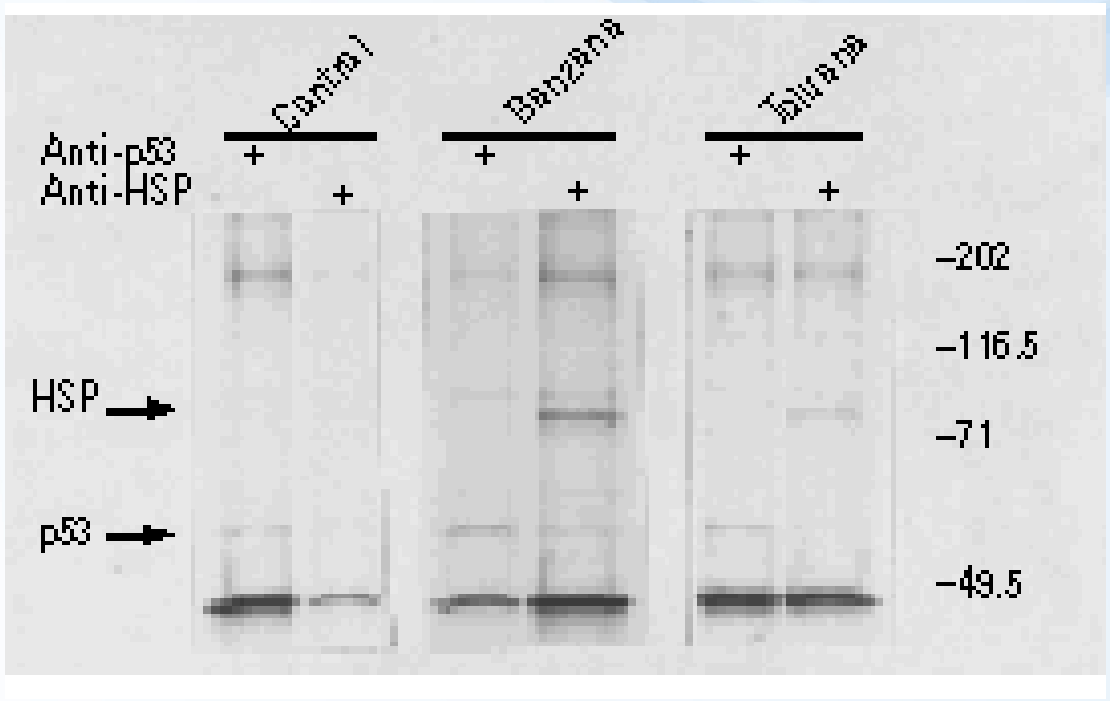
Figure 3-15

# 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

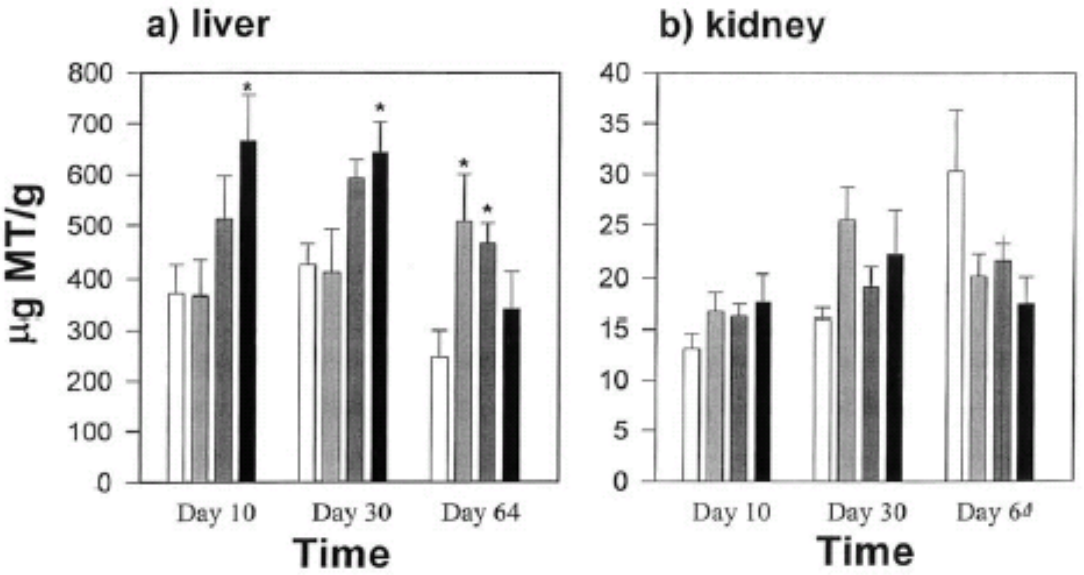



Fig. 2. Metallothionein (MT) concentrations in the (a) livers and (b) kidneys of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (±S.E.). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$).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

# Protein biomarkers of estrogenicity

**ER = transcription factor controling number of target genes**

Target genes of ER = biomarkers of estrogenicity

Major examples

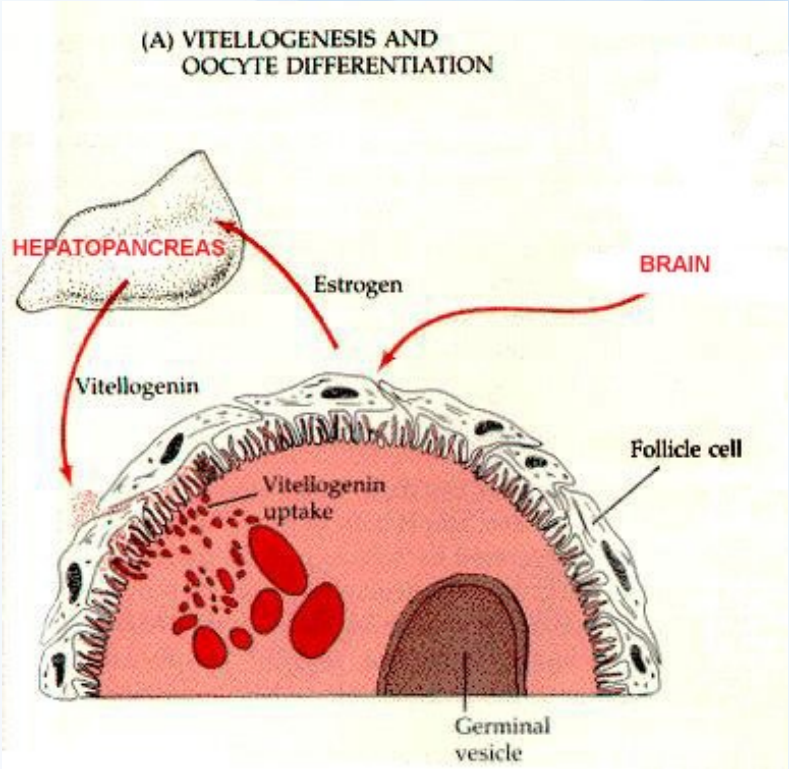
- **Vitellogenin**
- **Aromatase - CYP19A**

# Vitellogenin (Vtg)

Precursor of yolk proteins, phospho-protein („energy“ rich)
→ egg formations (females) at oviparous animals

Synthesized in liver and distributed via blood / haemolymph

**Xenoestrogens & other endocrine disruptors**
→ increased levels or early production in FEMALES
→ production de novo in MALES

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# Vitellogenin (Vtg) assessment

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

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

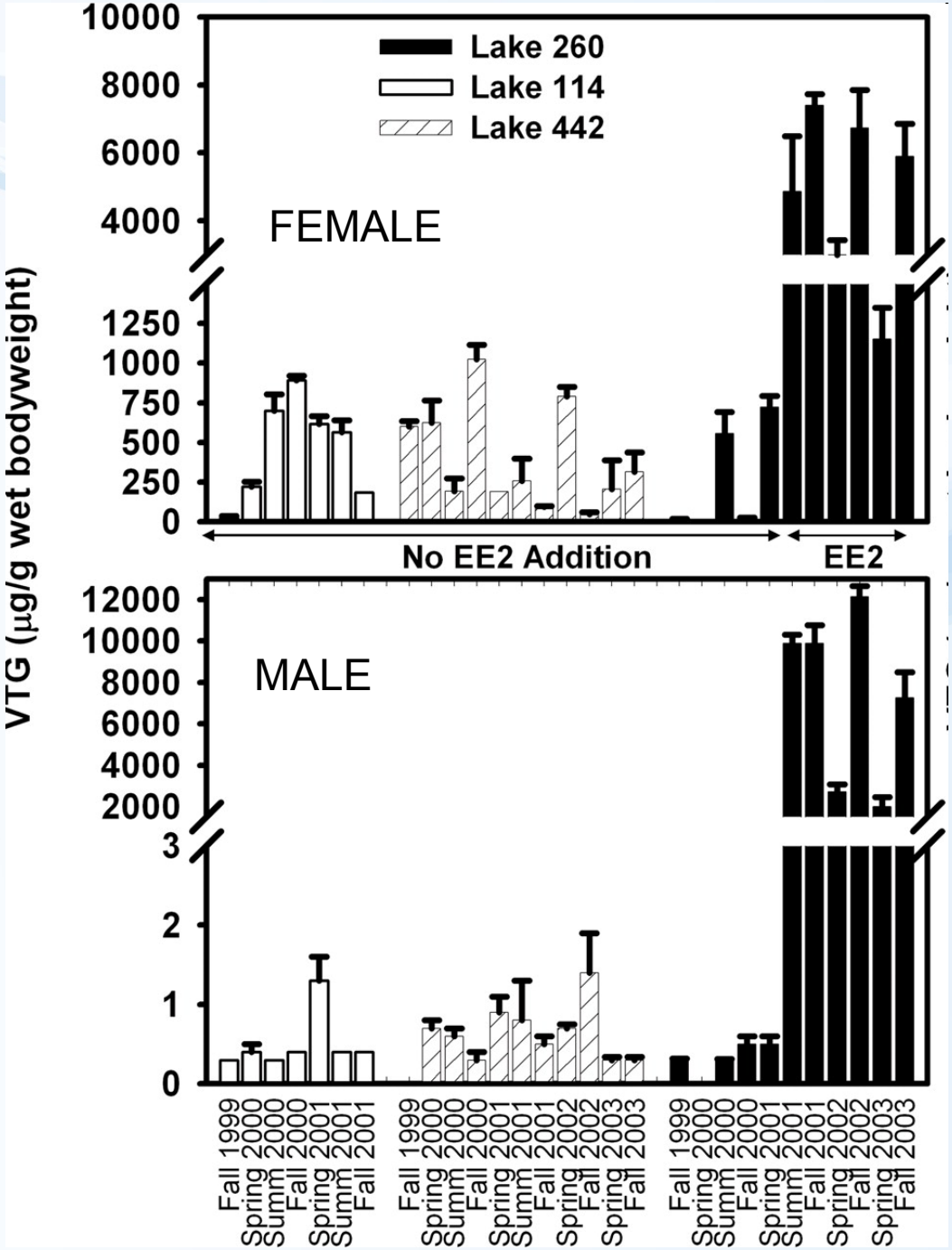
## 2) „Vitelin-like proteins“

- 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

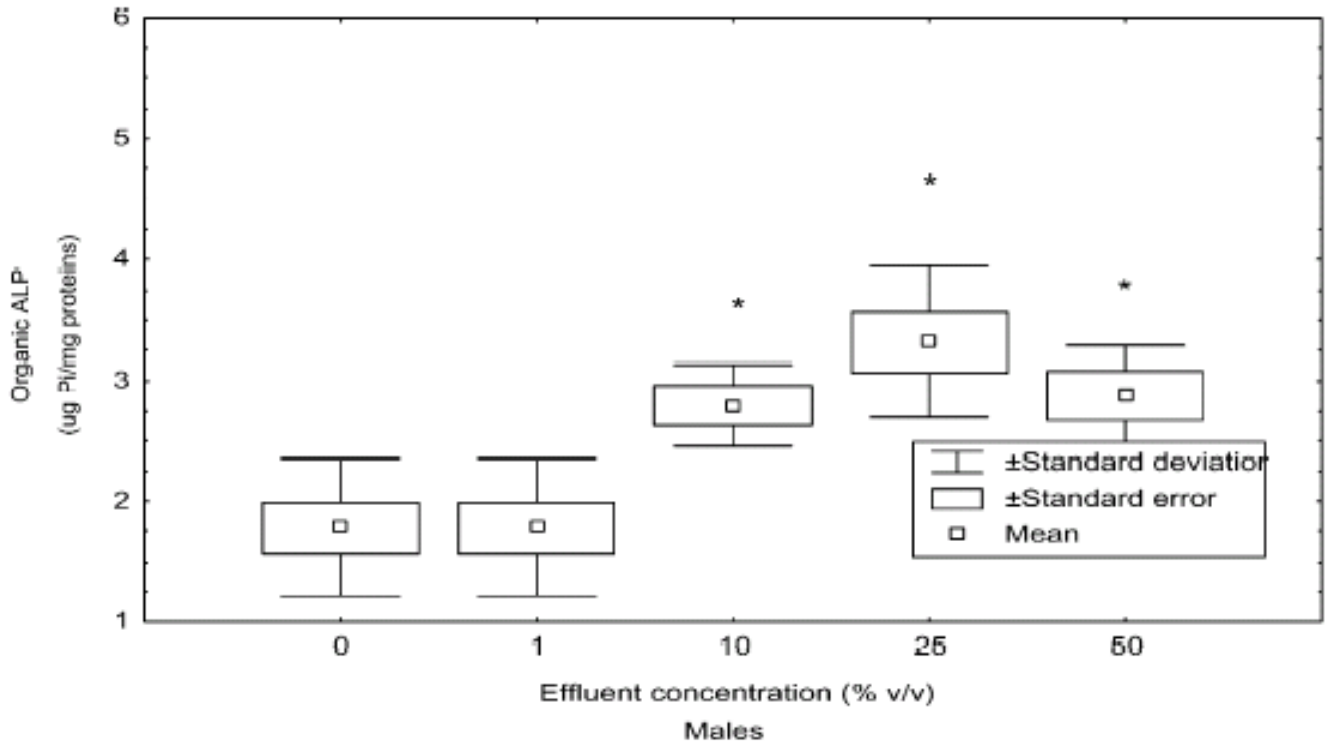
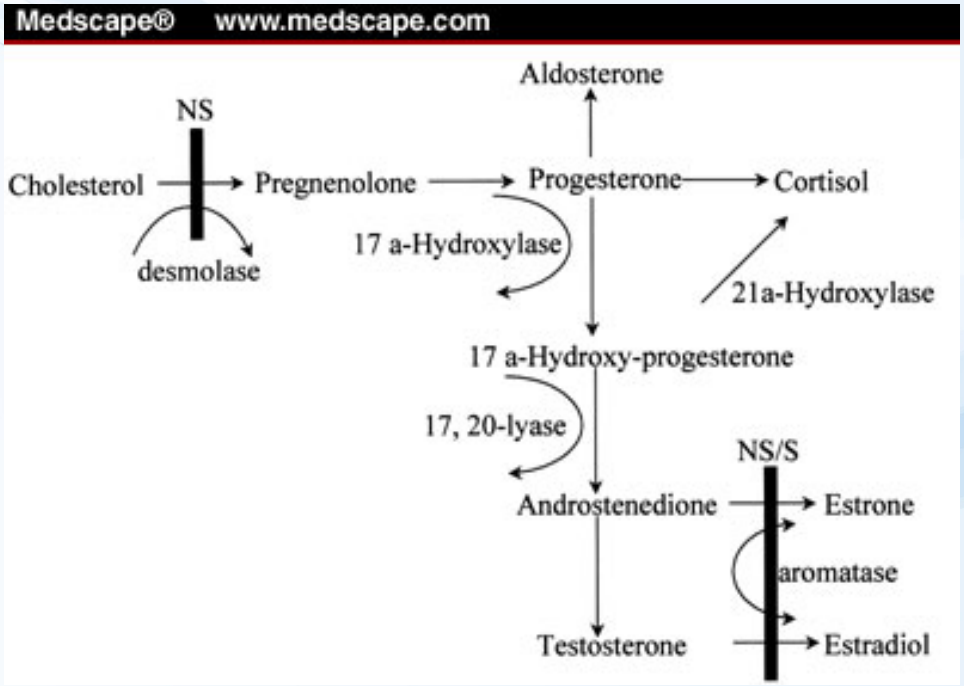



Fig. 4. Induction of Vg by exposure to a municipal effluent. Mussels were exposed for 96 h to a municipal effluent at 15°C. They were then collected for Vg and sex determinations. The asterisk (*) indicates significant difference at $P < 0.05$.

# Aromatase (CYP19A)

- Levels inducible by estrogens
- Catalyzes single enzymatic step
  **androgens → estrogens**

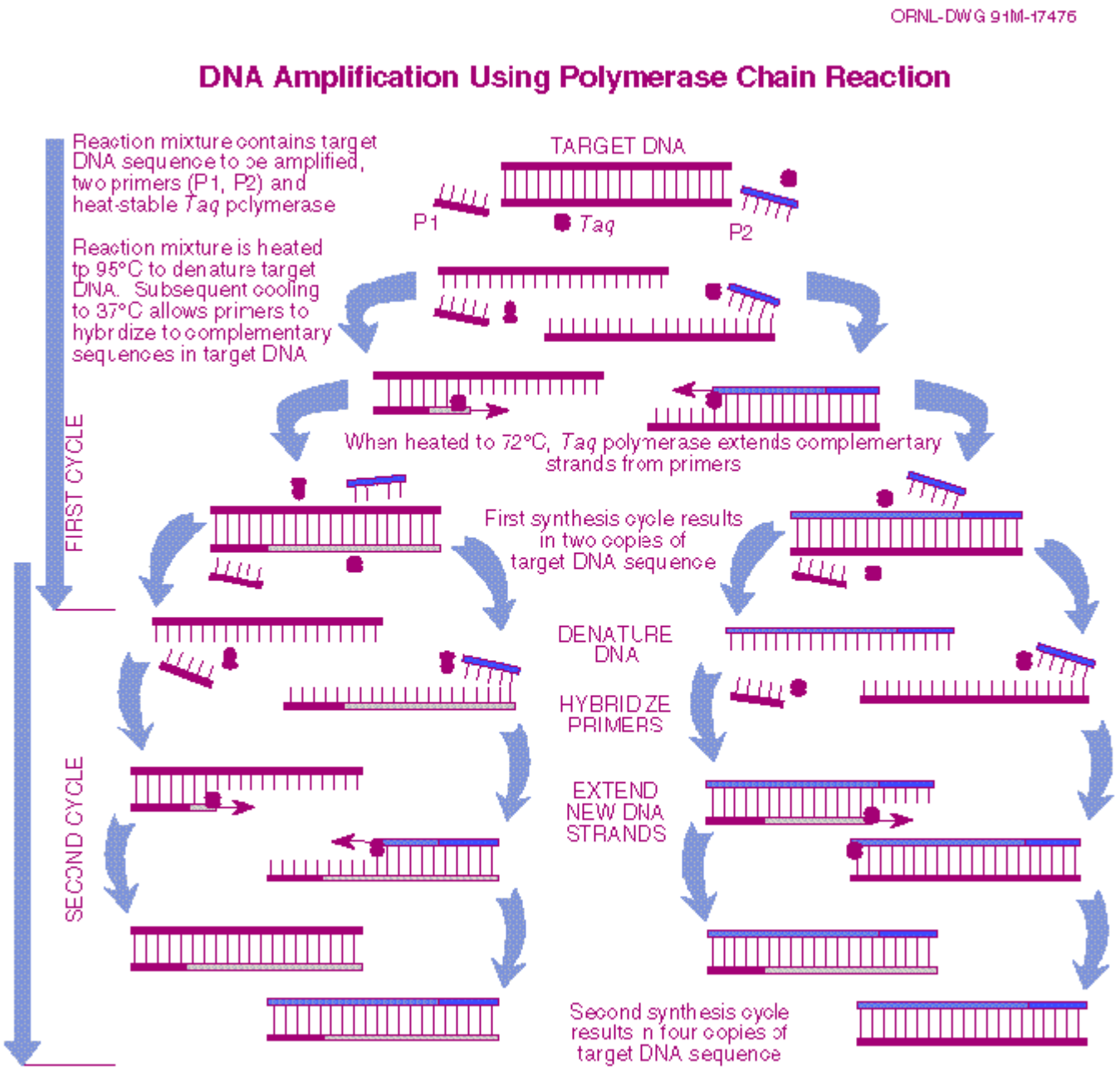



**Experimental assessment**
*(in reseach and practice)*

1. PCR / Quantitative-Real-Time-PCR

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

# PCR principle




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

# Visualization of PCR product

## 1) Electrophoresis (qualitative)

Intercalation dyes

– e.g. **ethidium bromide**

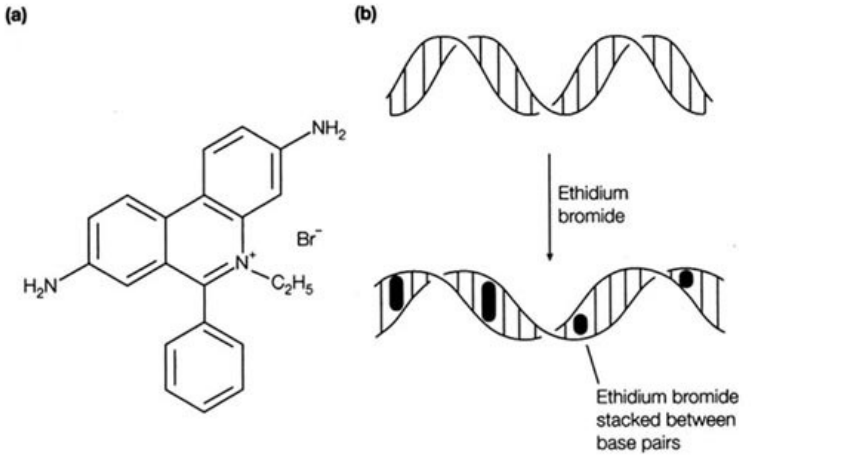
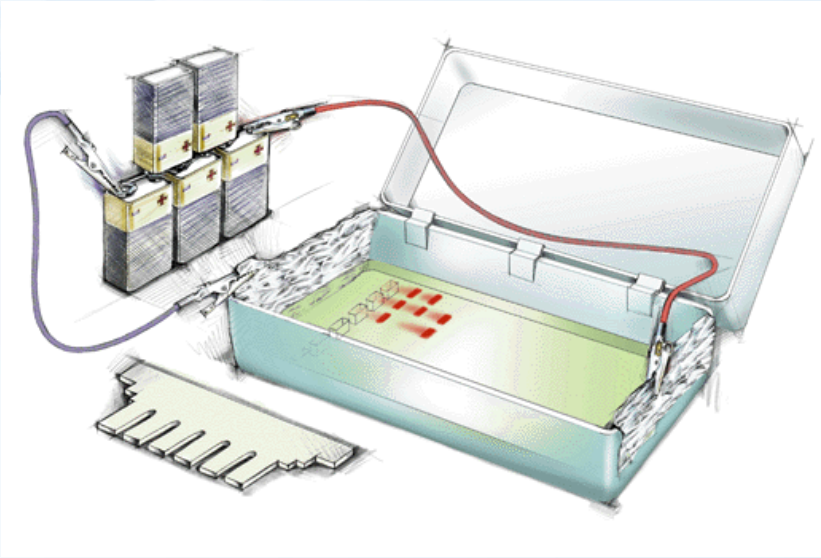
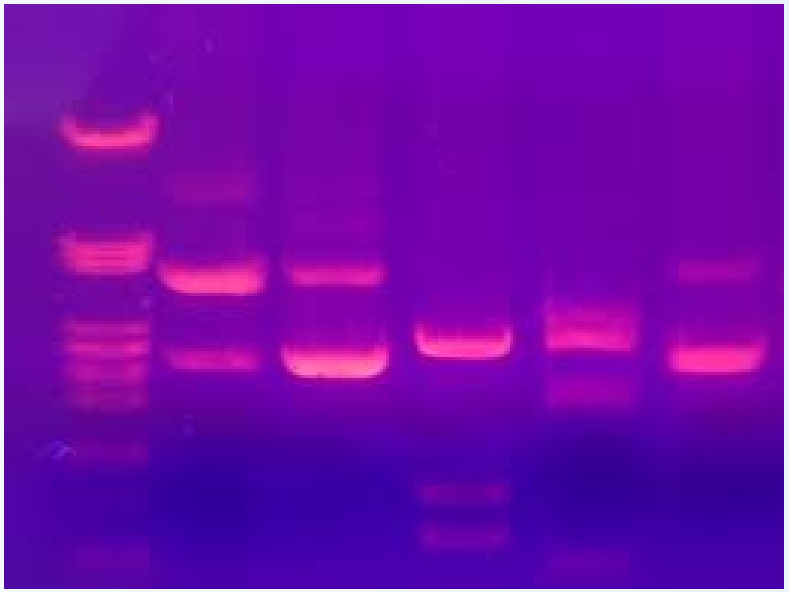



*Fig. 3. (a) Ethidium bromide; (b) the process of intercalation, illustrating the lengthening and untwisting of the DNA helix.*





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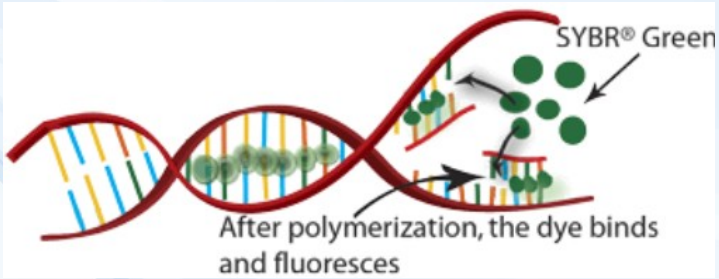
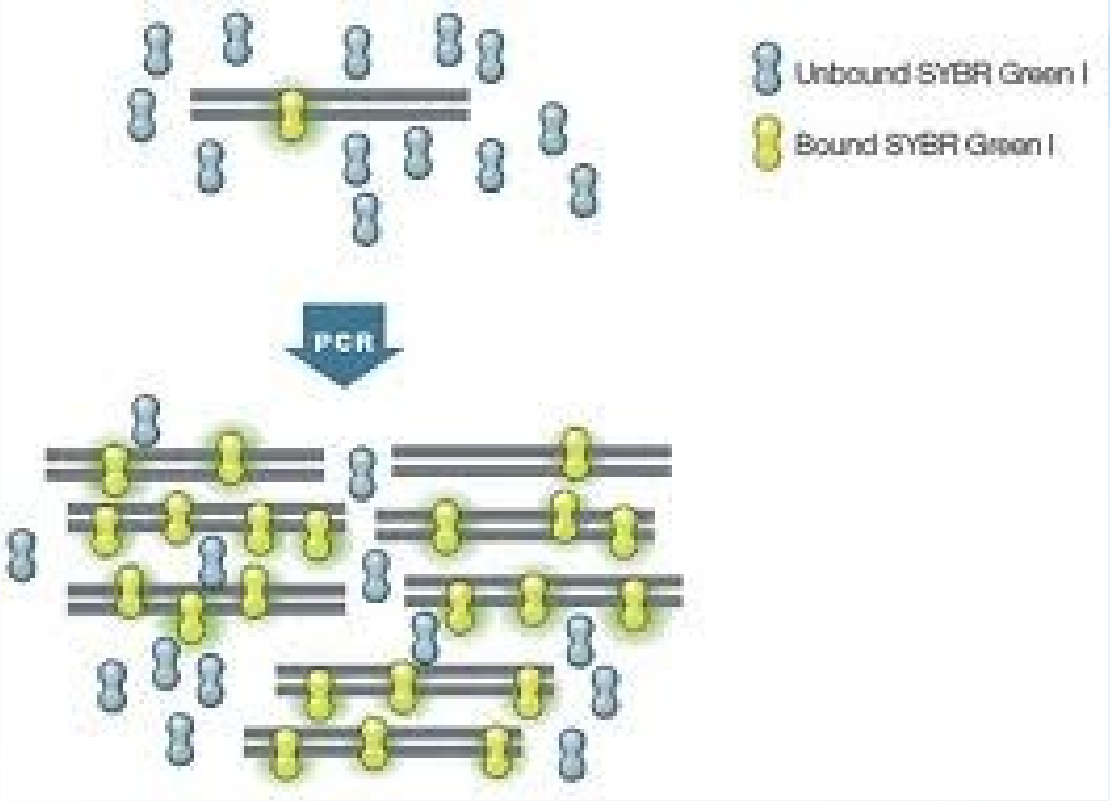
# Visualization of PCR product

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




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

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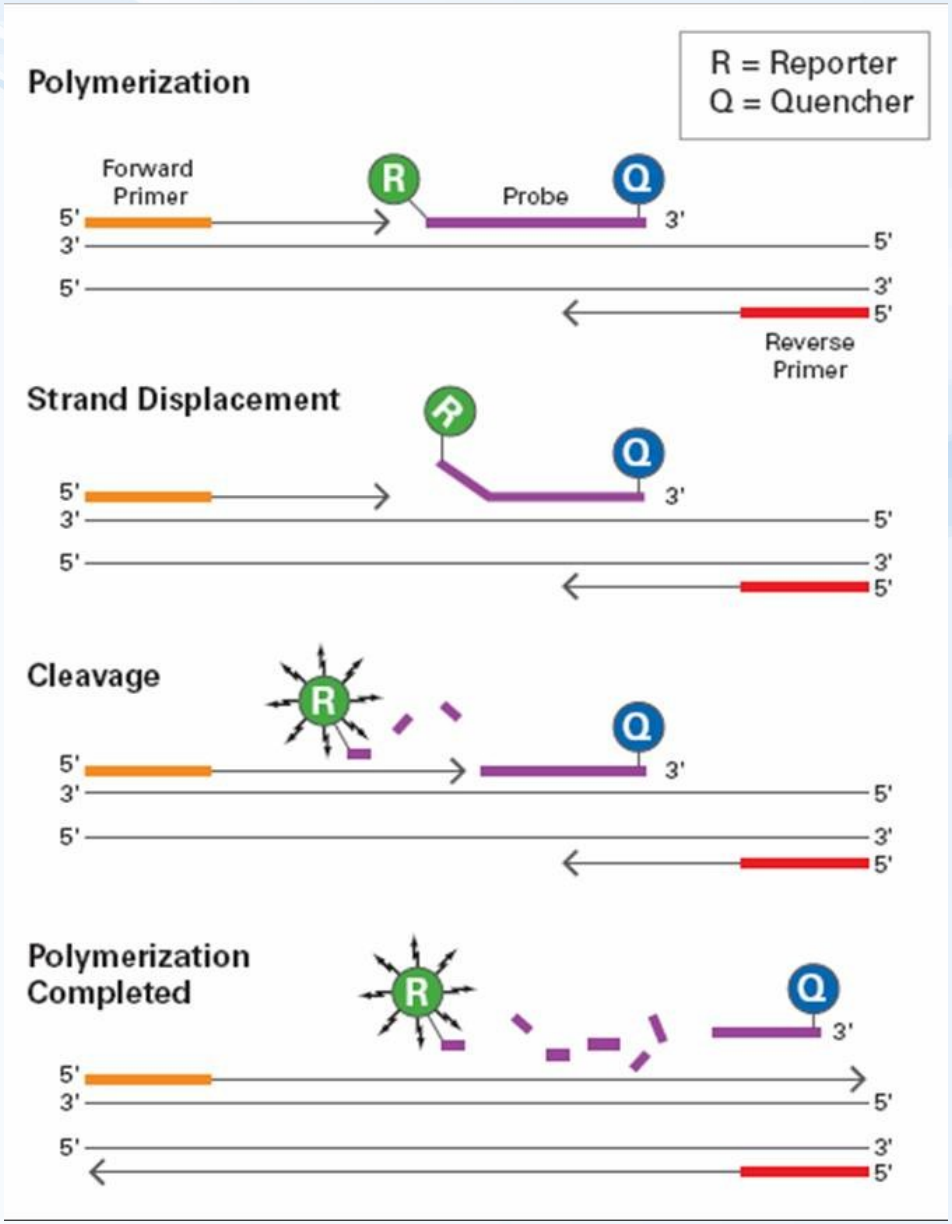
# Visualization of PCR product

## 2b) Real-time (quantitative) TaqMan probes

(more DNA replications
more fluorescent dye released)

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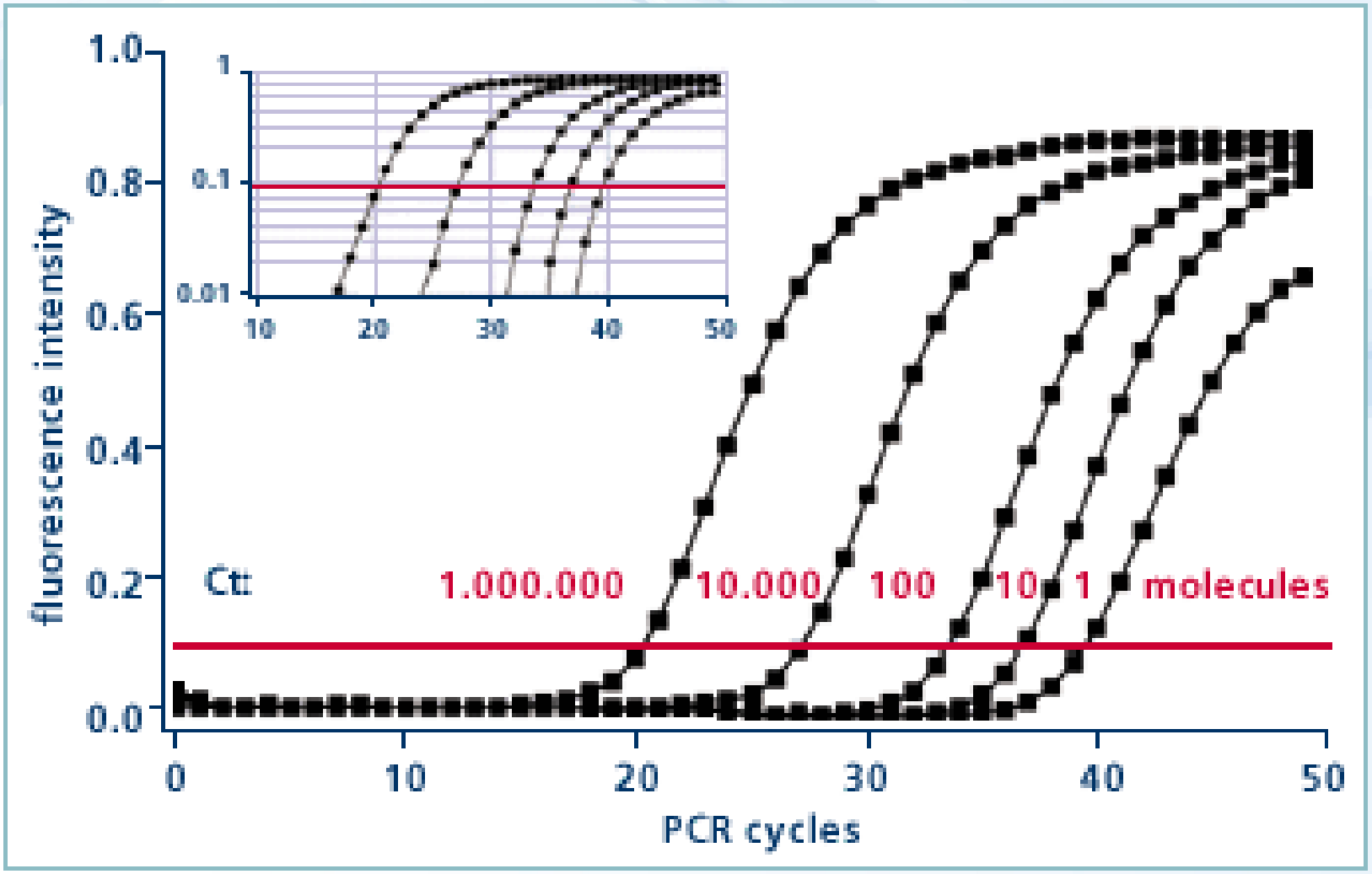
# Principle of “quantitative” PCR

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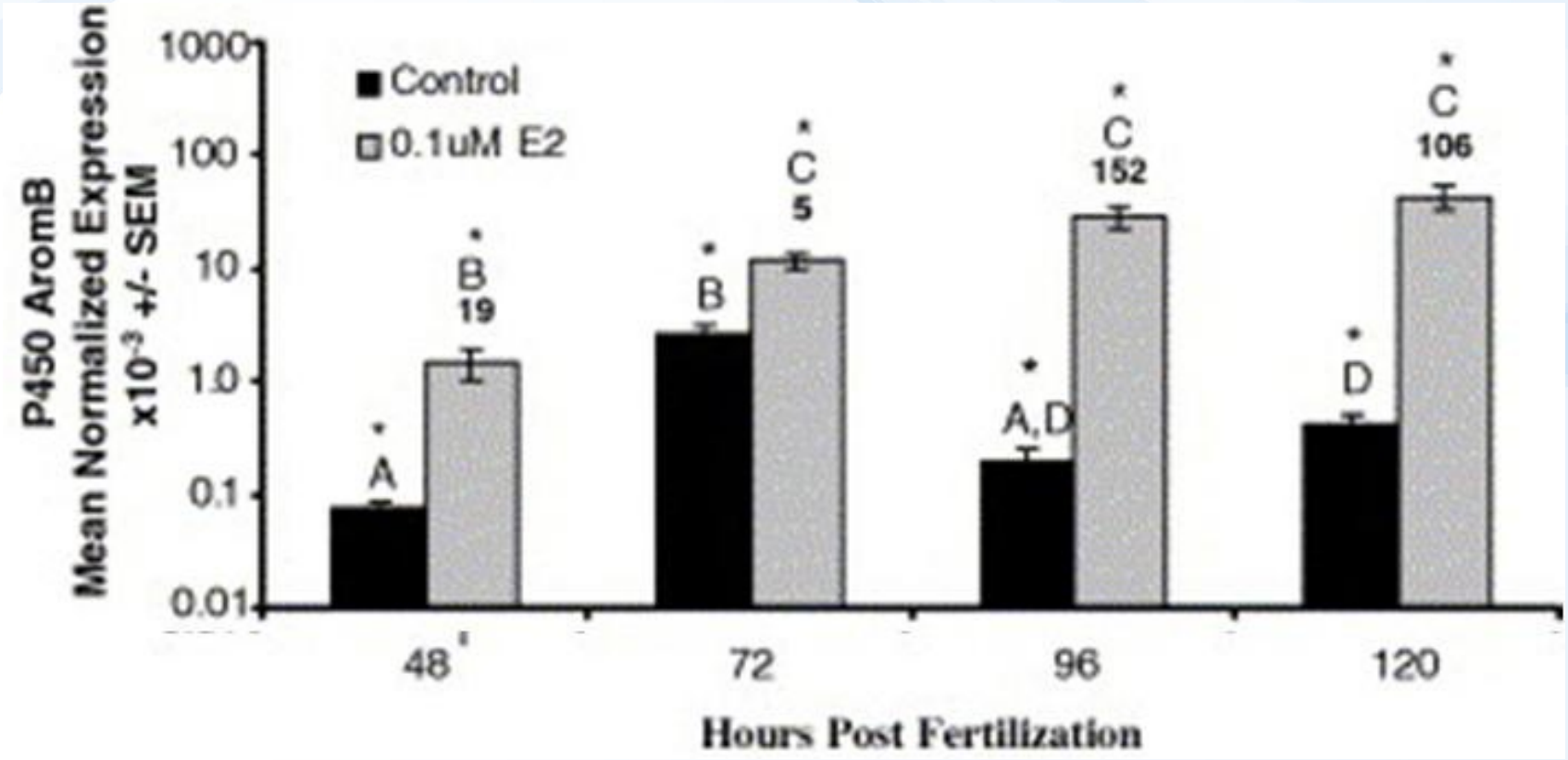
# qPCR determination of the aromatase gene in Zebrafish




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

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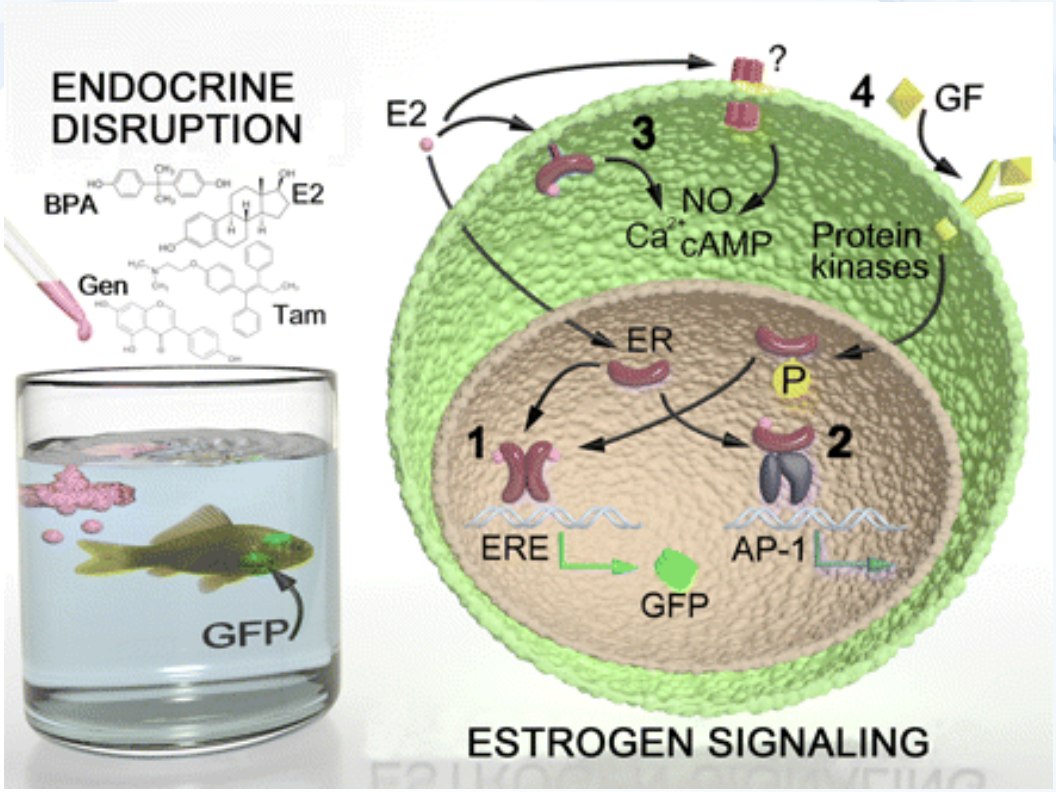
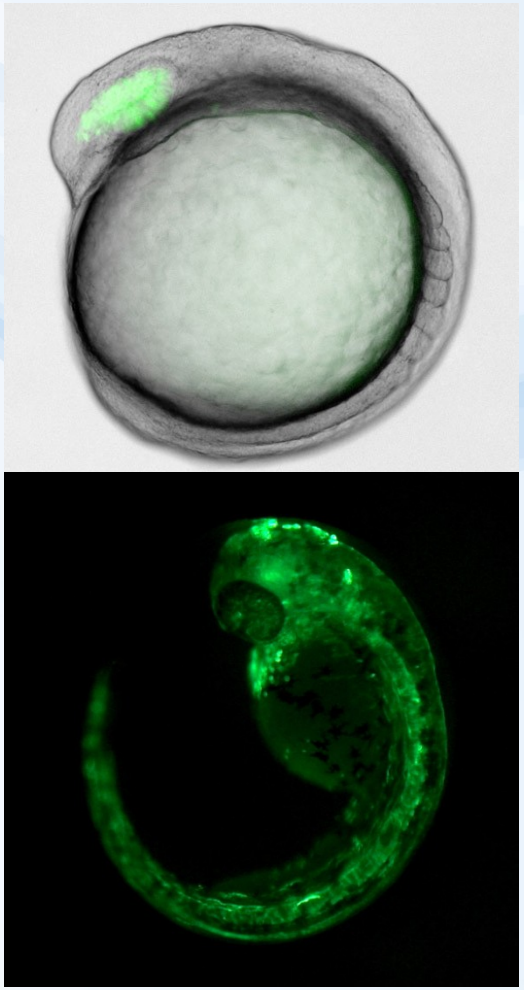
# GFP-reporter for estrogens in zebrafish embryo



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

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# Biomarkers of oxidative stress

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# 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)
- proteins: oxidized forms (carbonyls)

# Oxidative stress markers

## GSH

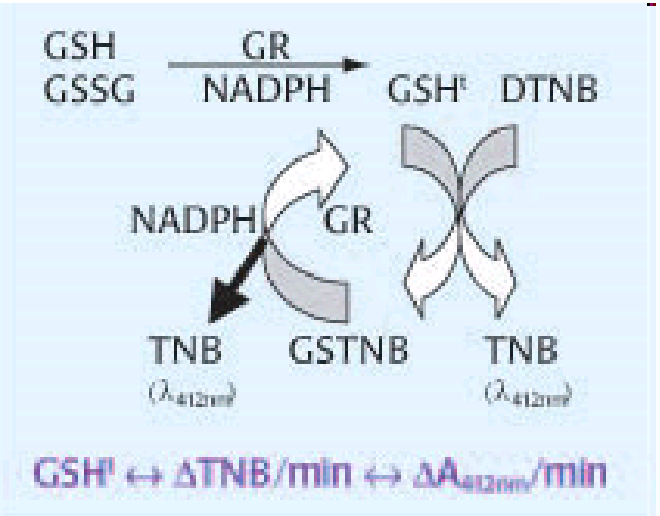
- antioxidant (scavenger of ROS) & reactive molecules
- conjugation molecules for detoxication
- probable intracellular regulatory molecule (? apoptosis ?)

**Total glutathione = reduced GSH + oxidized GSSG**

**Method of determination**

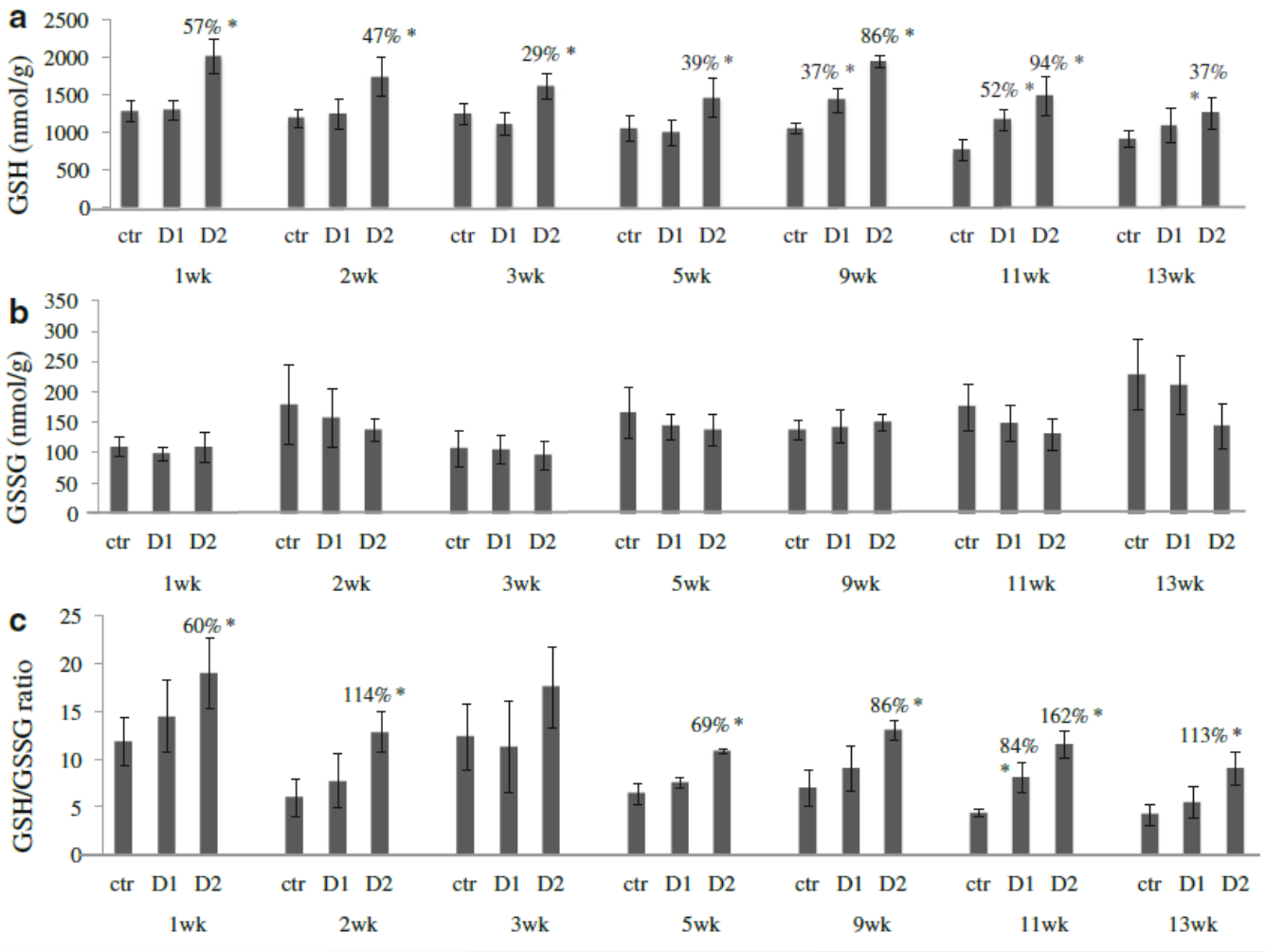
GSH + **Ellman s reagent (DTNB)** → Reduced GSH
GSH + GSH-reductase + **DTNB** → Total GSH

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

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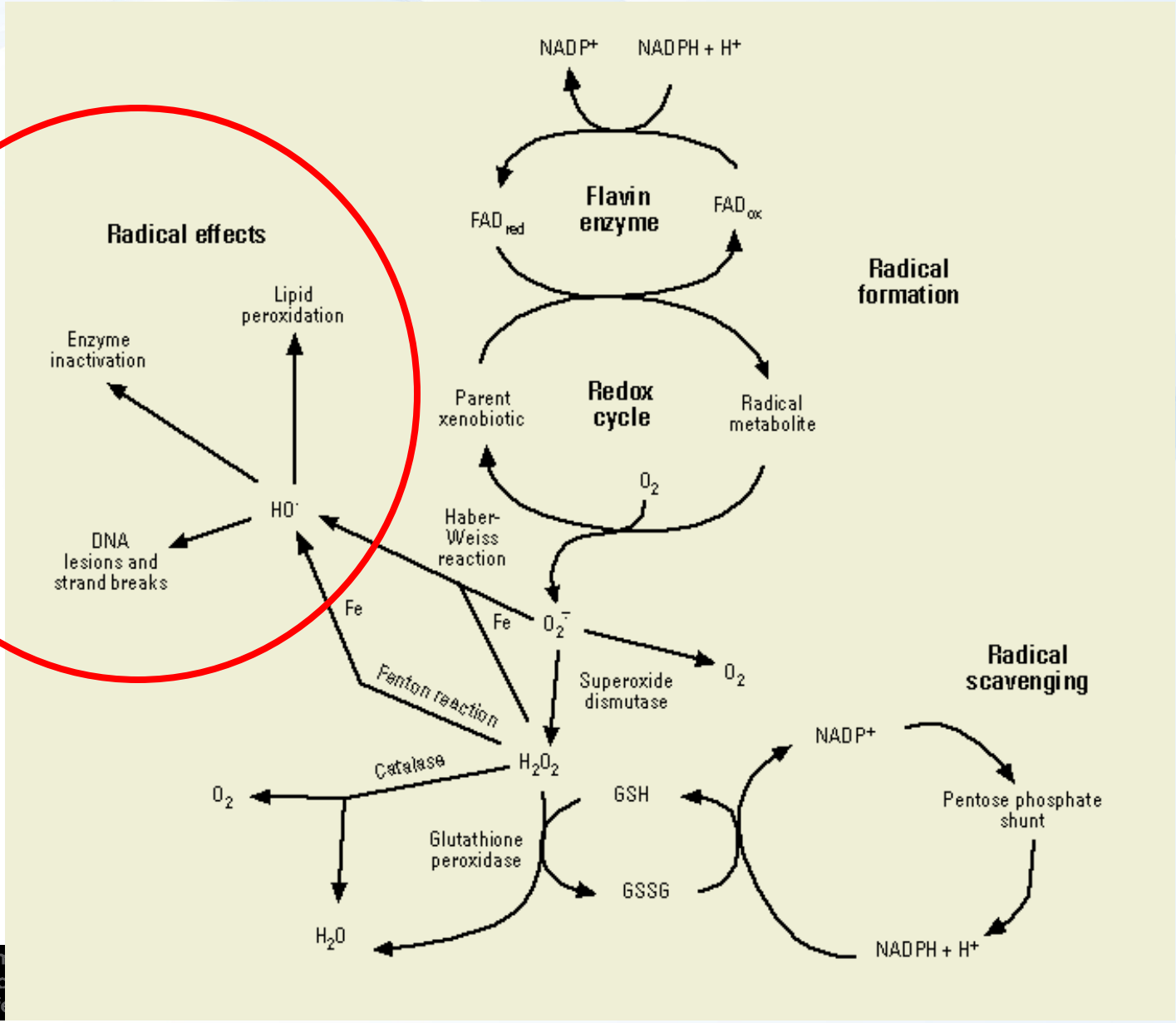
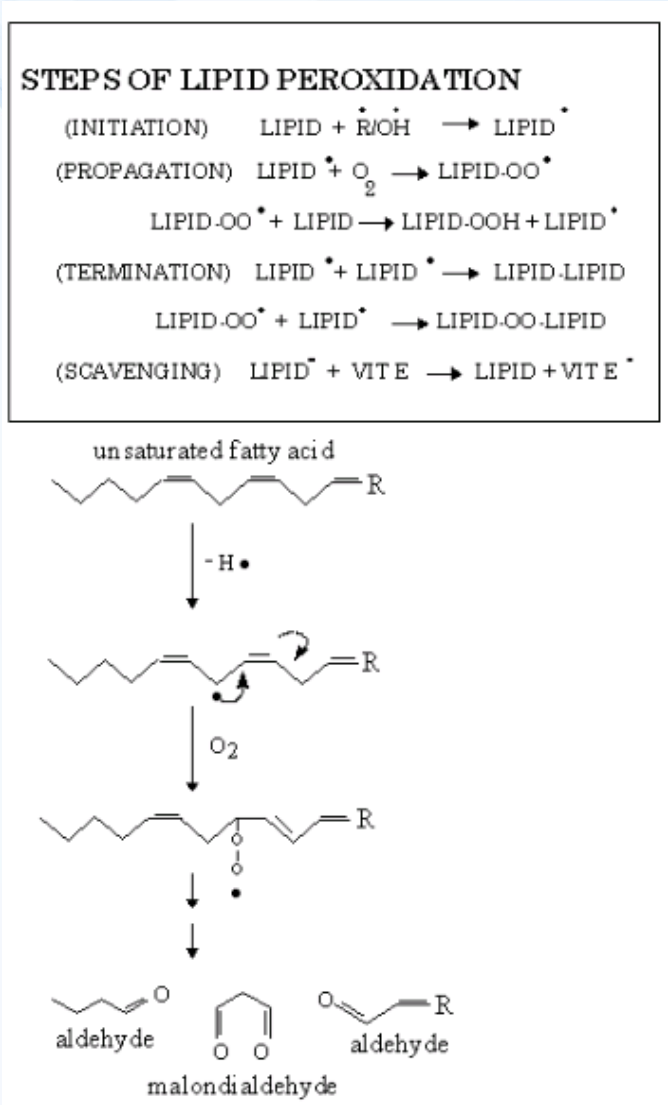
# Markers of oxidative DAMAGE



# Lipid peroxidation → Malondialdehyde (MDA)

**MDA – malondialdehyde**

**product of lipid peroxidation**

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# Malondialdehyde (MDA) determination

**MDA – formed from oxidized membrane phospholipids**

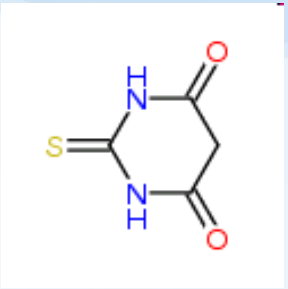
: determination:

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

**TBARS – ThioBarbituric Acid Reactive Species**

: less specific than HPLC

: easy determination (spectrophotometry)




TBA

**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)

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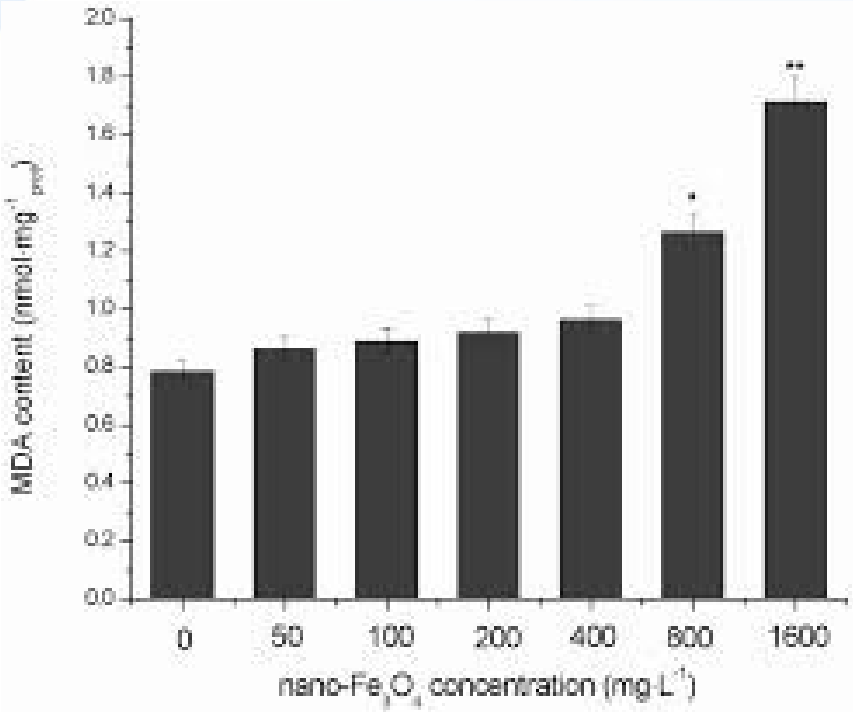
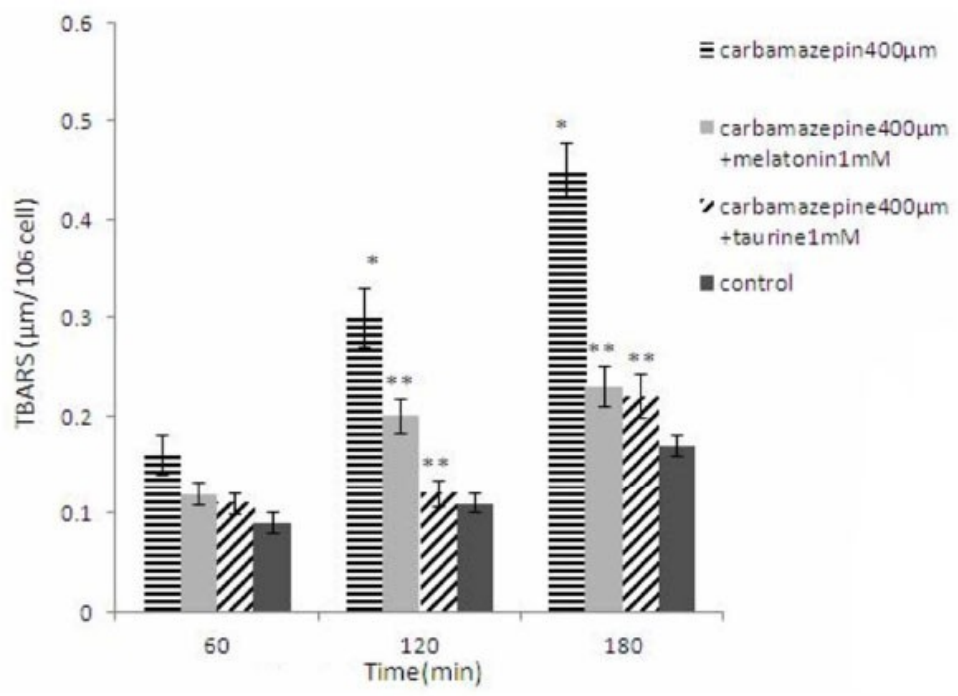
# MDA modulation - examples

Effects of nanoFeOxide particles on MDA in fish




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

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