In vivo biomarkers of effects / response

Do we know the agent ? Do we expect the effect ?
: specific biomarkers / non-specific changes

Behavioral and Clinical biomarkers

Pathology

Clinical chemistry and hematology

Enzymatic changes

Protein synthesis biomarkers

Oxidative stress markers

Behavioral and clinical biomarkers

Behavioral and clinical biomarkers

Parameters evaluated

- body weight
- food consumption
- fitness & welness

Interpretation

: are these ? biomarkers ?
(effects already demonstrated in vivo)

- biomarkers of existing serious stress / intoxication

Behavioral and clinical 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).

(Histo)pathology biomarkers

Pathology

(-) Destructive methods, Time consuming, Professional requirements

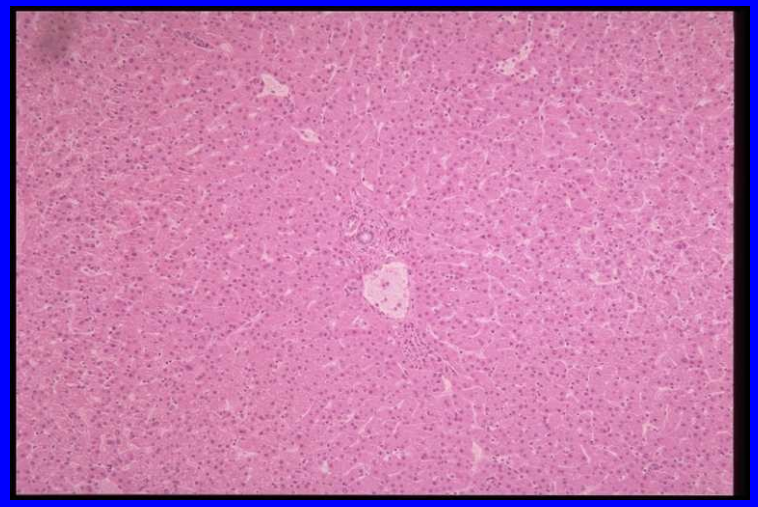
(+) High relevance – organ/tissue changes

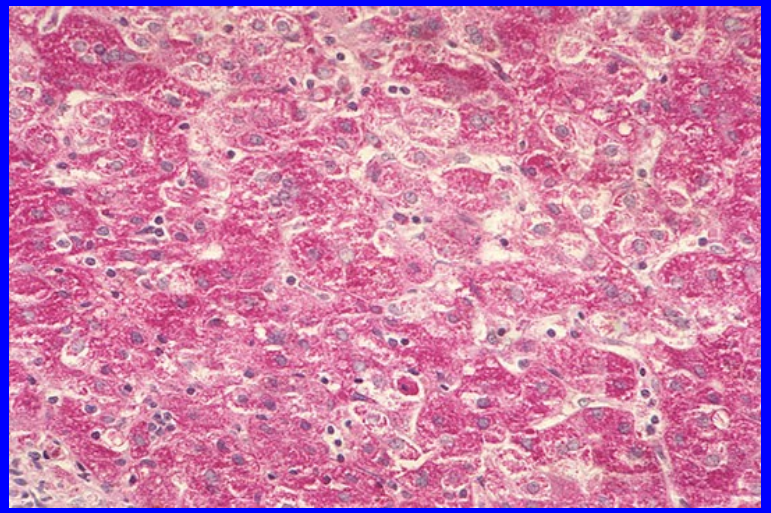
1) microscopy of internal organs

: non-specific changes in internal organs

: specific **changes in liver** (dioxin-like POPs, cyanobacterial toxins ..)

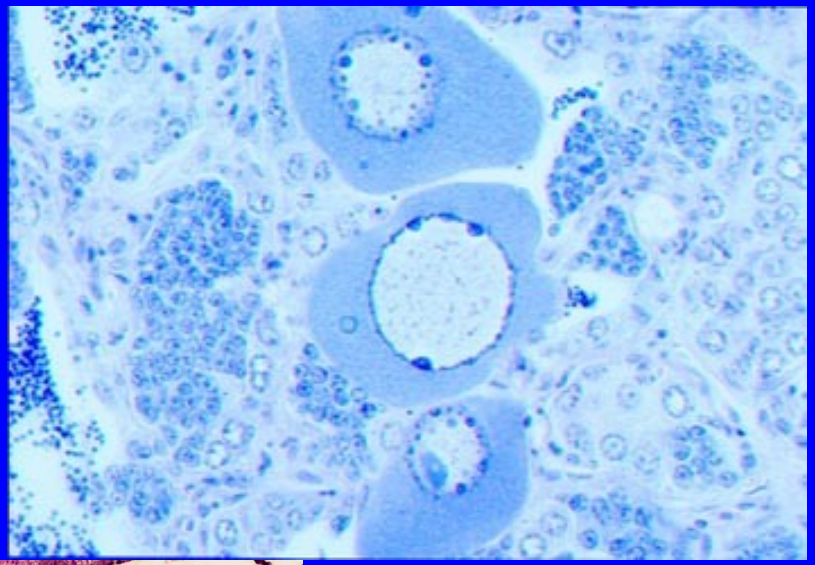
: **intersex / imposex formation** (xenoestrogenicity)



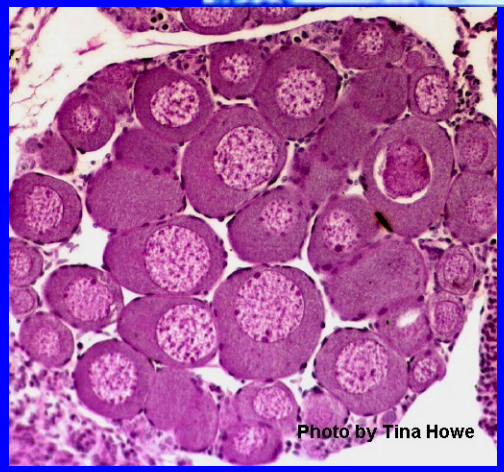


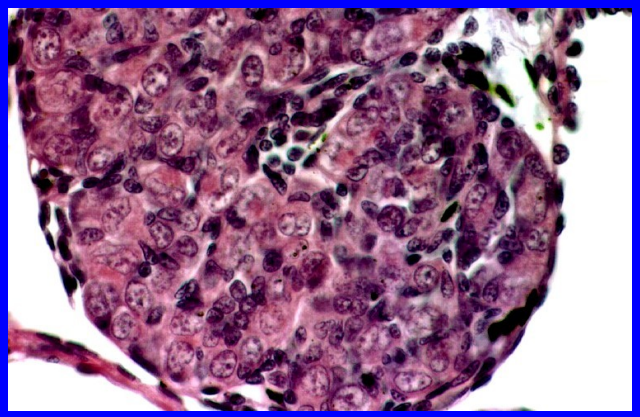
Example: Liver damage by cyanobacterial toxins microcystins

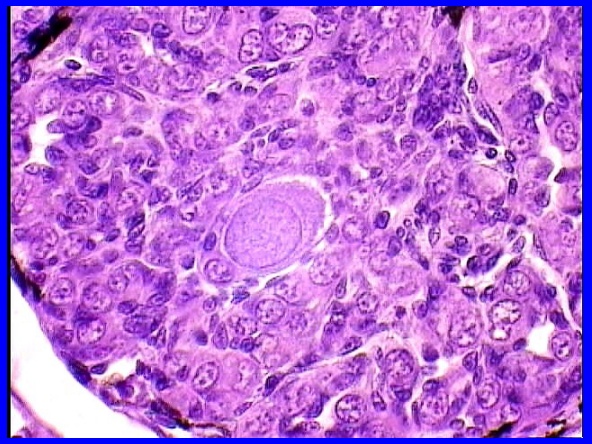
Endocrine disruption: Intersex microscopy



Oocytes
in testicular tissue







Pathology

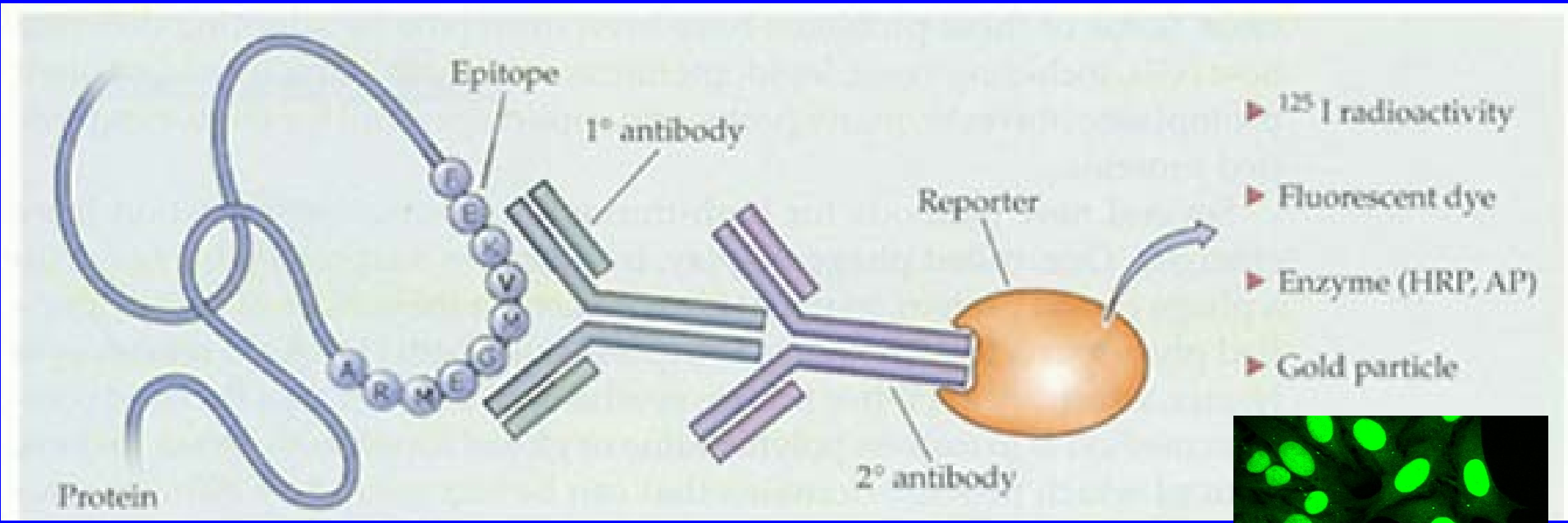
2) immunohistochemistry & microscopy

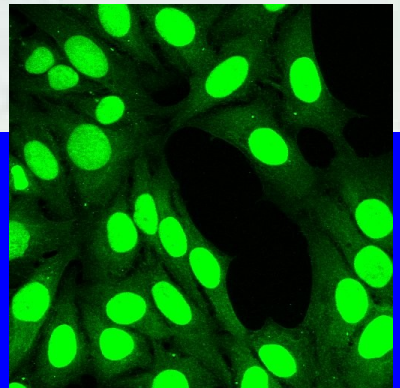
: determination of specific changes

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

- toxicant induced autoimmunity:

anti-nuclear Ab, ANA





Pathology

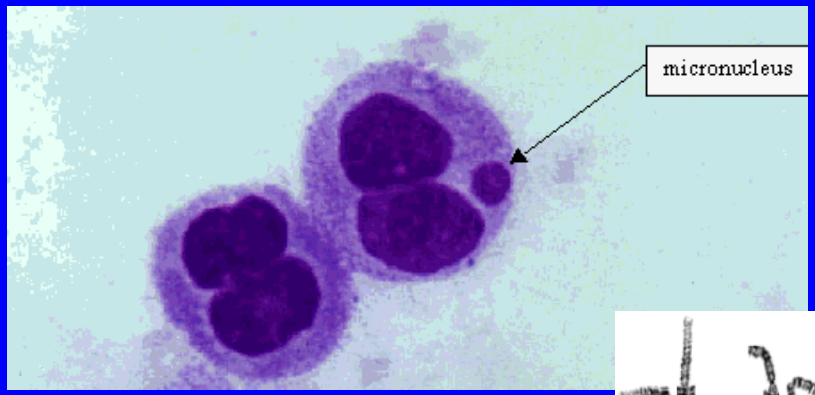
3) Nuclear DNA characterization

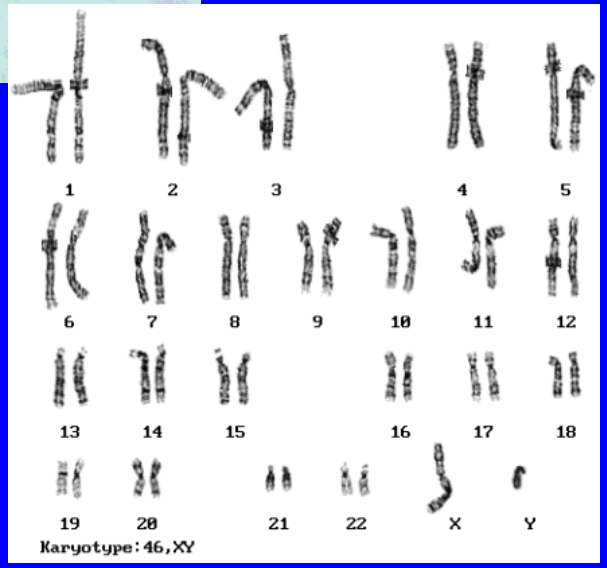
- micronuclei evaluation

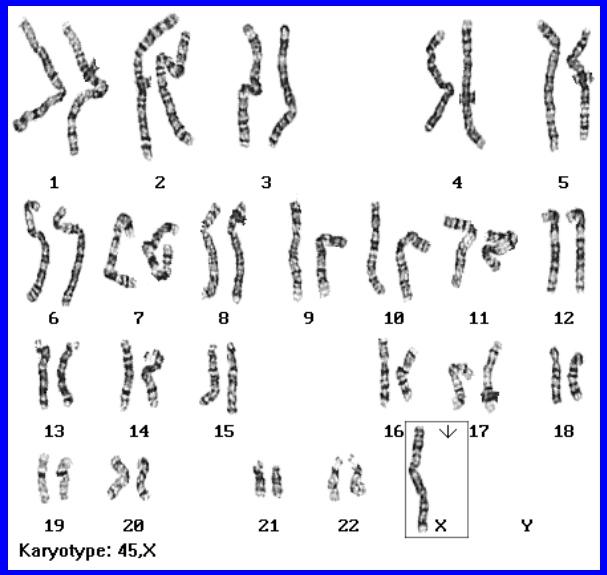
- chromosomal abnormalities :

karyotype biomarkers (*human genetic disorders)*

: non-destructive (blood samples; plant tissues)







Clinical chemistry & hematology biomarkers

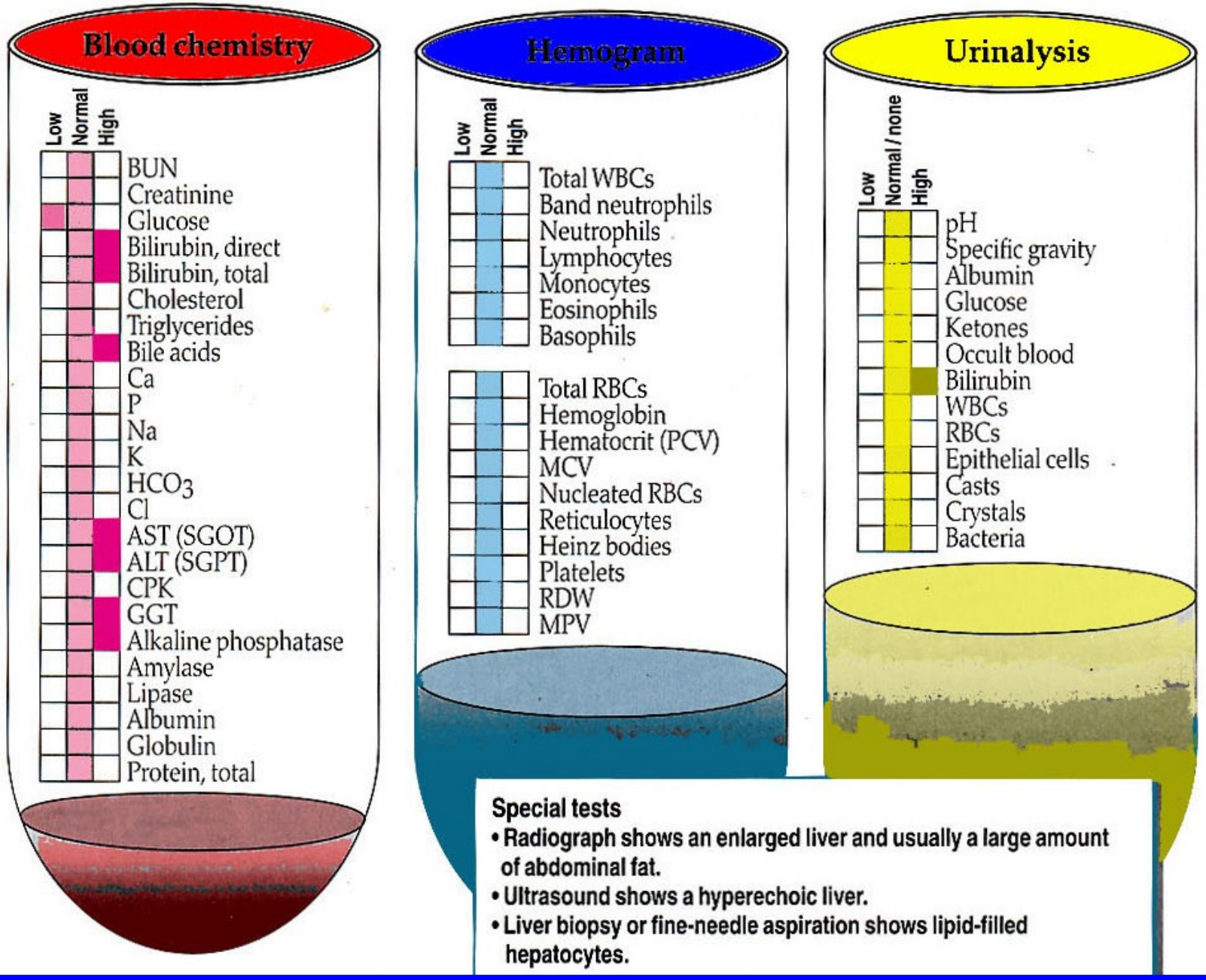
Clinical chemistry & hematology

Non-destructive (BLOOD, URINE sampling)

<u>Multipe parameters</u> 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 chemistry
Low
Normal
High
BUN
Creatinine
Glucose
Bilirubin, direct
Bilirubin, total
Cholesterol
Triglycerides
Bile acids
Ca
P
Na
K
HCO3
Cl
AST (SGOT)
ALT (SGPT)
CPK
GGT
Alkaline phosphatase
Amylase
Lipase
Albumin
Globulin
Protein, total
Hemogram
Low
Normal
High
Total WBCs
Band neutrophils
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils
Total RBCs
Hemoglobin
Hematocrit (PCV)
MCV
Nucleated RBCs
Reticulocytes
Heinz bodies
Platelets
RDW
MPV
Urinalysis
Low
Normal / none
High
pH
Specific gravity
Albumin
Glucose
Ketones
Occult blood
Bilirubin
WBCs
RBCs
Epithelial cells
Casts
Crystals
Bacteria
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.

Clinical chemistry & hematology

Methods:

- automatic biochemical and hematological analyzers
- different „analytes“ various principles of methods

Clinical chemistry & hematology

Often with specific interpretation:

- determination of enzymatic activities in blood

- tissue/organ-specific damage damage

Examples *(toxicological studies)*

- **liver damage** – **AST** (Aspartate aminotransferase), **ALT** (Alanine aminotransferase) in blood…
  : cyanotoxins, dioxin-like POPs

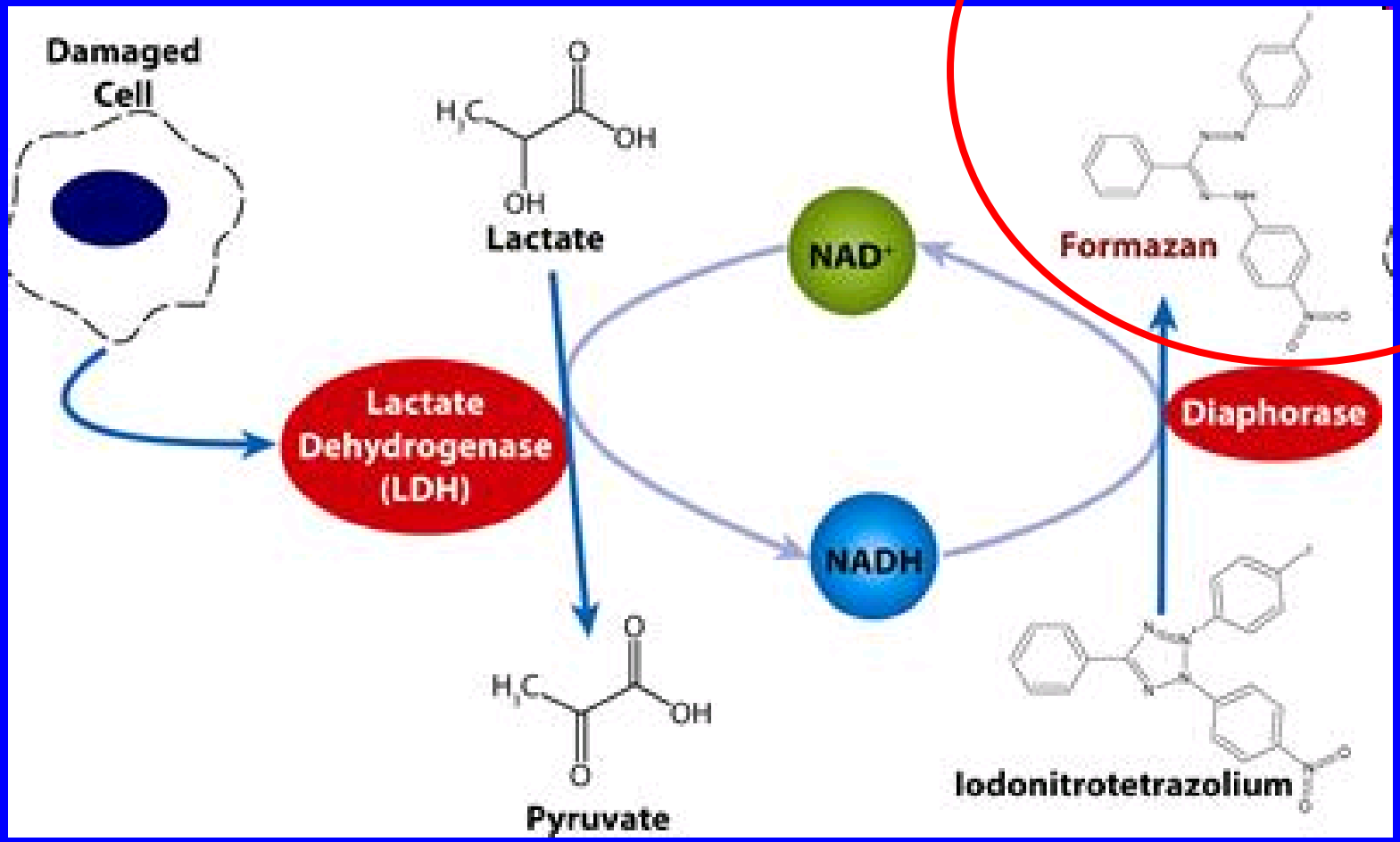
- ***lactate dehydrogenase*** *(LDH) - general cell damage*

- *muscle damage:* ***creatine kinase*** *in serum*
  : isozymes - tissue specific (brain, muscle, heart);

Clinical chemistry & hematology

LDH assay - principle

Kinetic Spectrophotometry



Example – changes in rat serum enzymes after CCL4 exposure

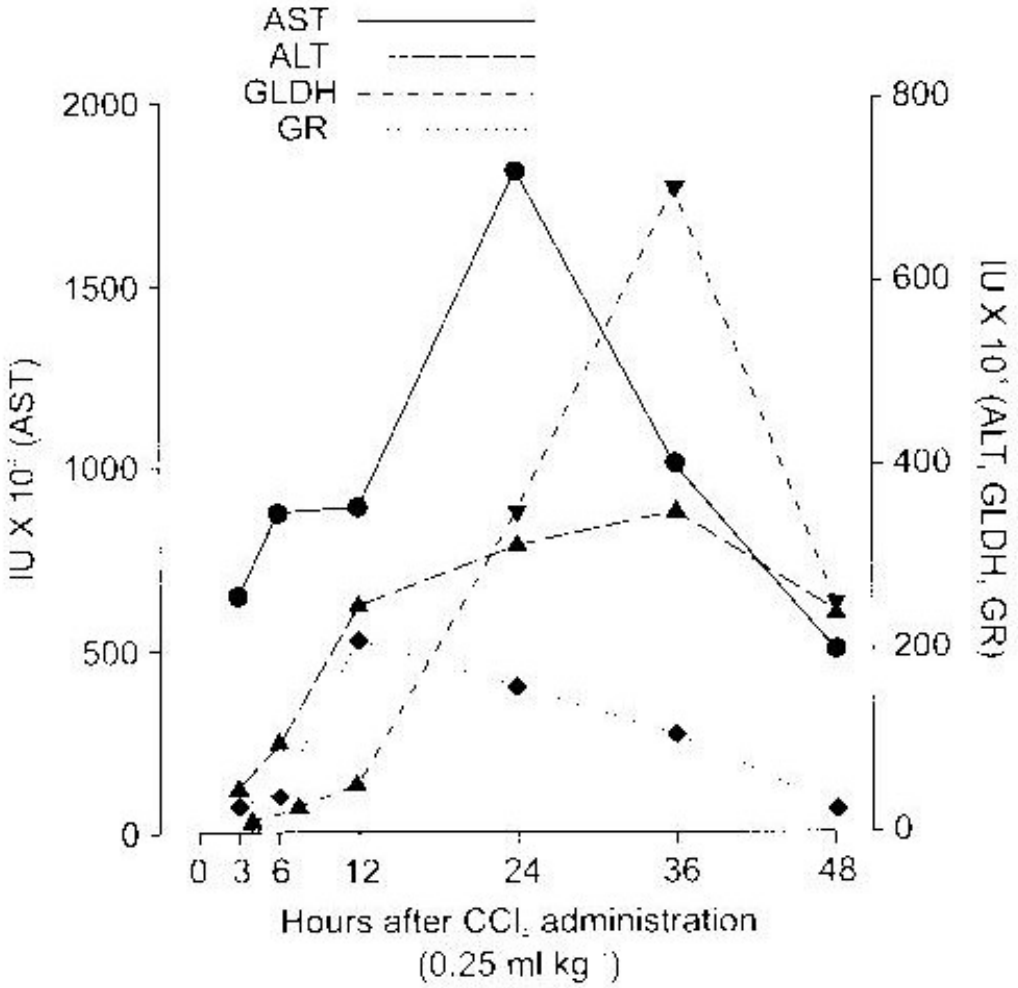


Figure 3 Serum enzyme levels in rats following dosing with carbon tetrachloride (CCl_4, 0.25 ml kg^{-1}). Redrawn from Zimmerman (1978).

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)

Clinical chemistry & hematology

+ Human: Excretory products in urine

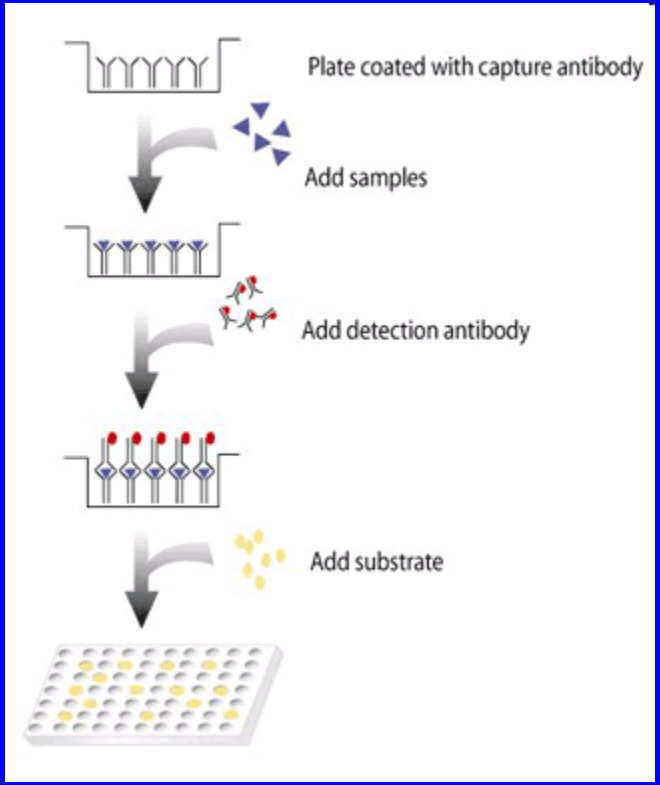
Tumor genes and tumor markers

- 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

Toxicity mechanisms related to „enzyme changes“:

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

AcChE inhibition mechanism

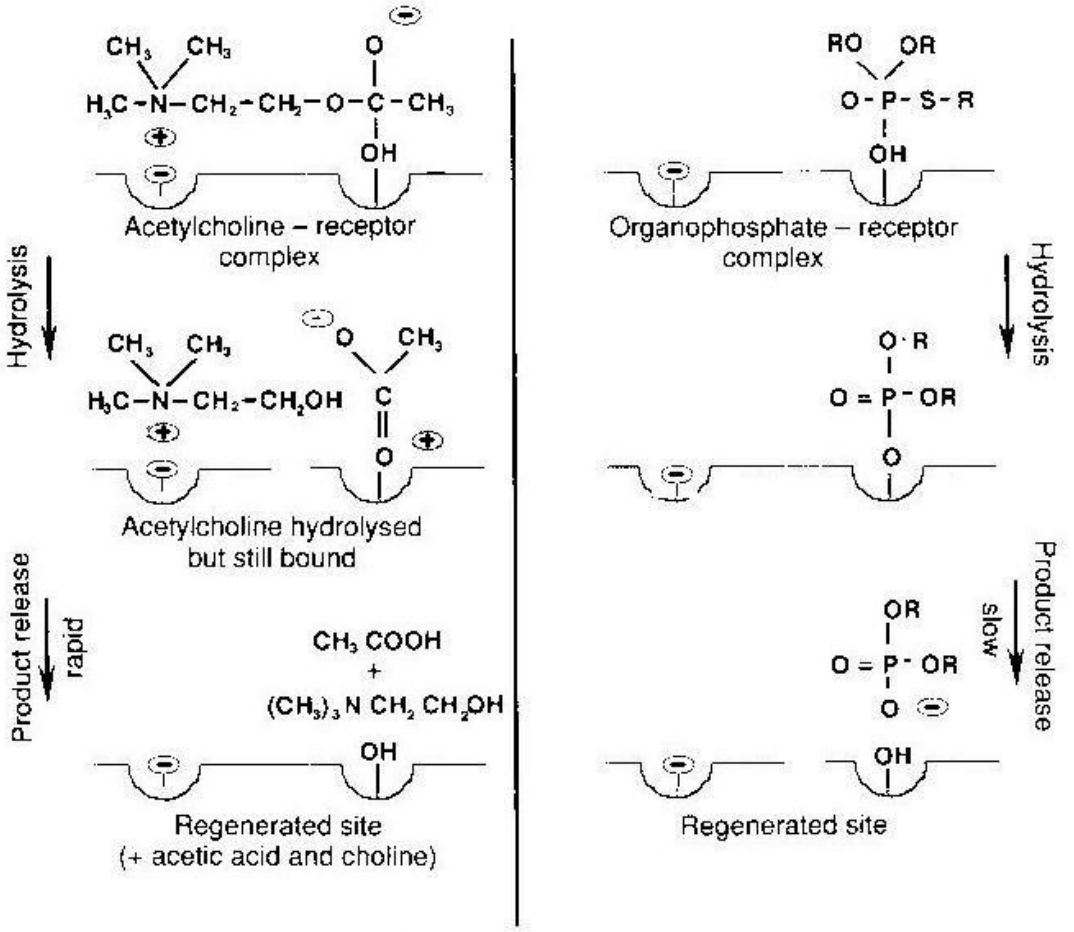
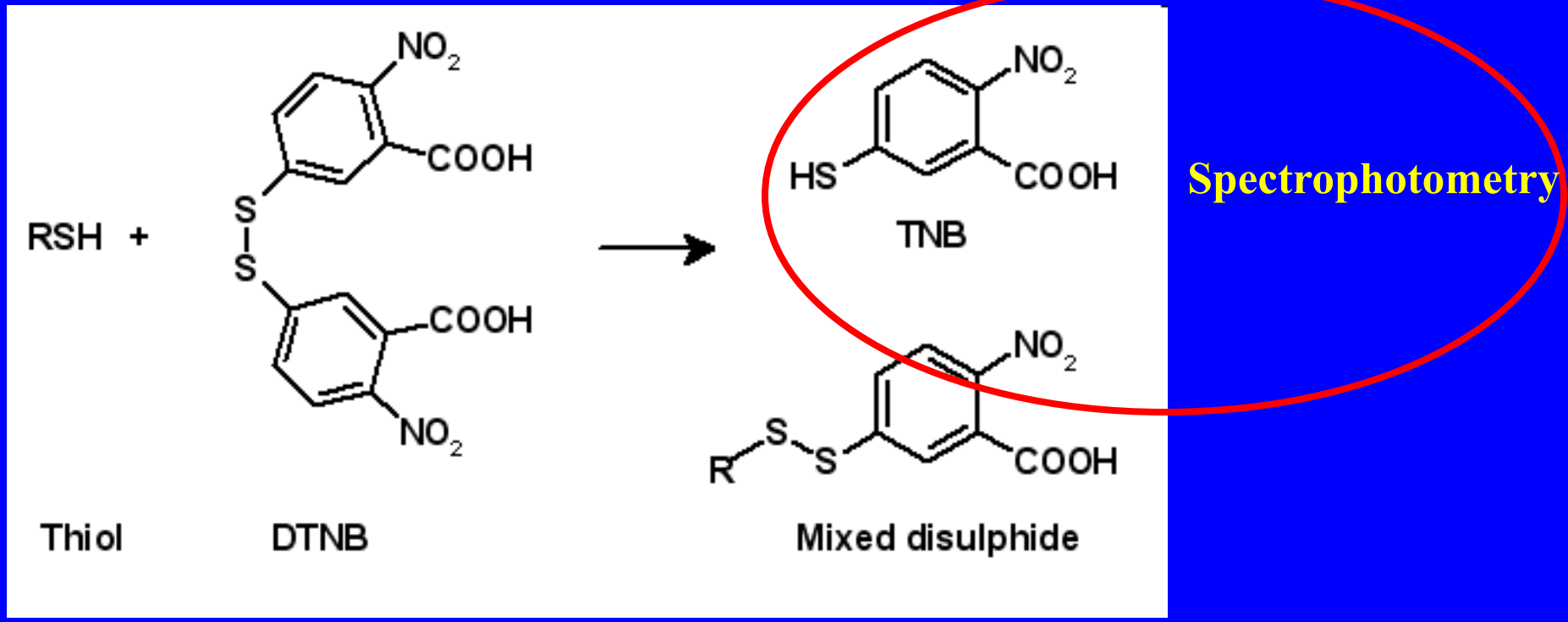


Figure 2.2 Mode of action of inhibition of acetylcholinesterase.

AcChE inhibition assay

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

- cleaved by **AcChE** → formation of free –SH groups
- **SH: thiol reactive probes: Ellman´s reagent (DTNB)**
- DTNB-S-choline: yellow colour (spectrophotometry A420)



AcChE inhibition
mechanism & effects in birds

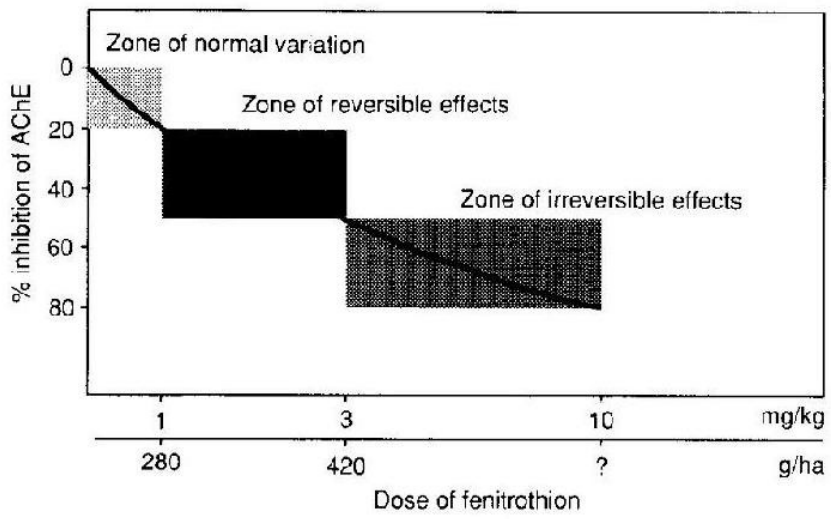


Figure 10.2 Dose response of AChE inhibition.

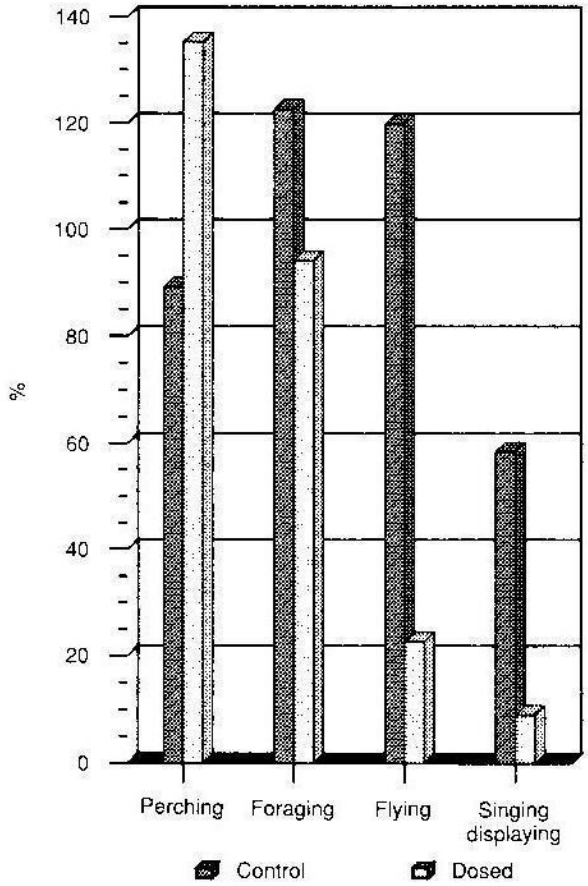


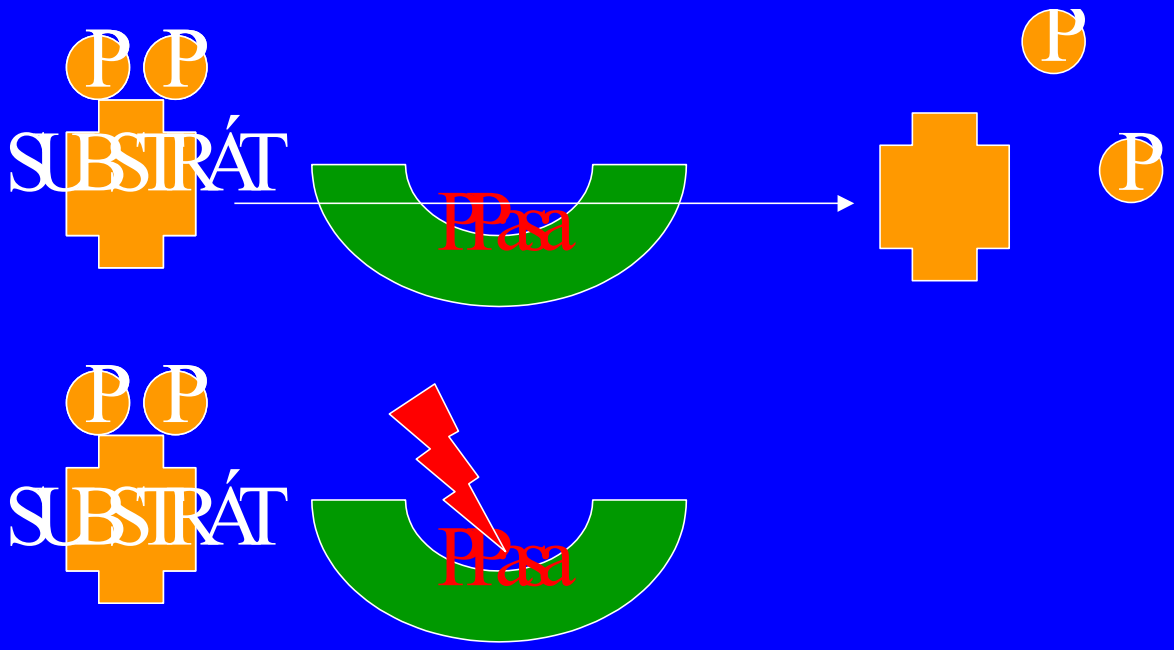
Figure 2.4 Effect of OP on behaviour of starlings. After Grue and Shipley (1981).

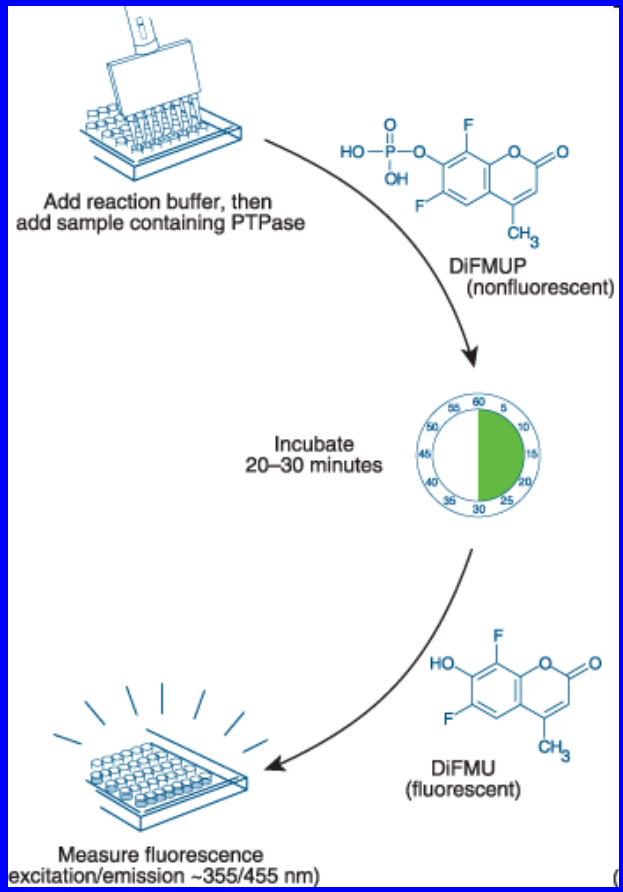
Proteinphosphatase inhibition assay

Model substrates cleaved by PPase

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

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





MFO (CYP) activities

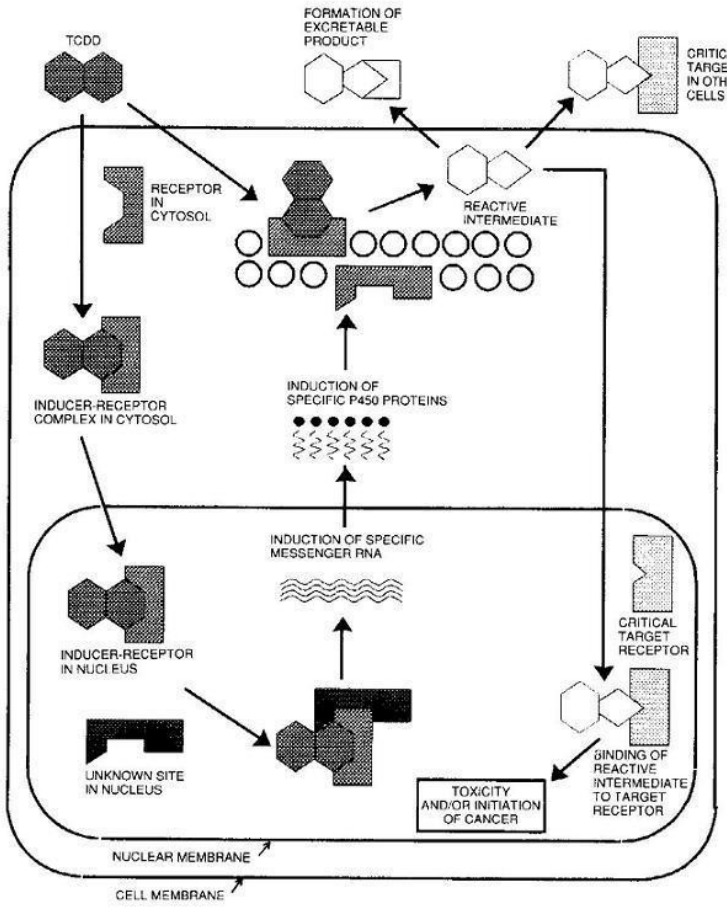


Figure 5.1 Diagram of MFO system. Nebert and Gonzalez (1987).

Table 5.1 Classification of P450s

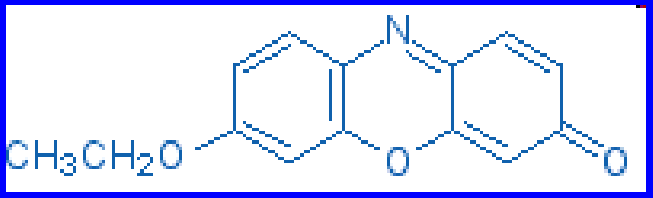
Nomenclature	Induced by/specificity
P450I	Polycyclic aromatic, TCDD
P450II	Phenobarbital-inducible family*
P450IIA	Specific for testosterone hydroxylase
P450IIB	PB inducible
P450IIC	PB inducible
P450IID	Specific for debrisoquine 4-hydroxylase
P450IIE	Ethanol inducible
P450III	Steroid inducible
P450IV	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).

MFO (CYP) activities

EROD assay

- Determination of CYP450 activity



substrate: Ethoxyresorufin

-> Oxidation by CYP1A1 -> Fluorescence

EthoxyResorufin-O-Deethylase activity EROD

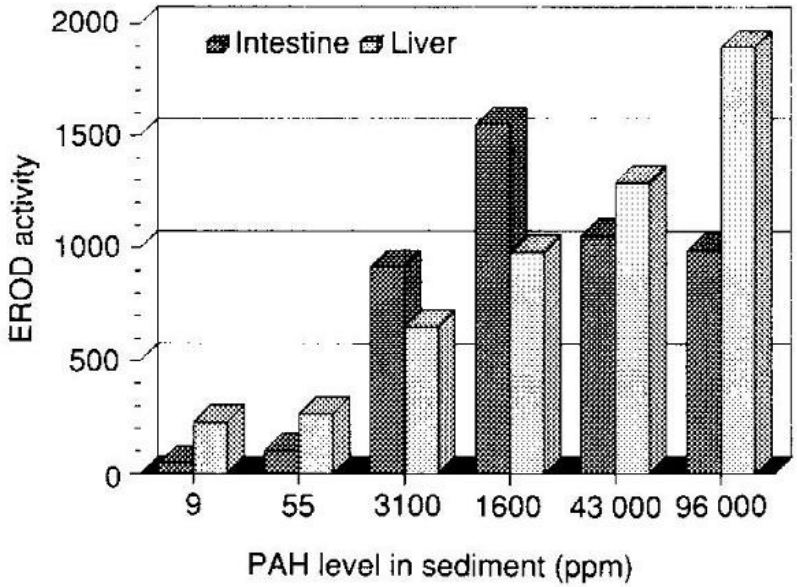
(other substrates: CYP isozymes:

BROD - butoxy…, MROD, PROD …)

Biomarker of organic pollution (exposure & effects)

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

: often used in environmental studies



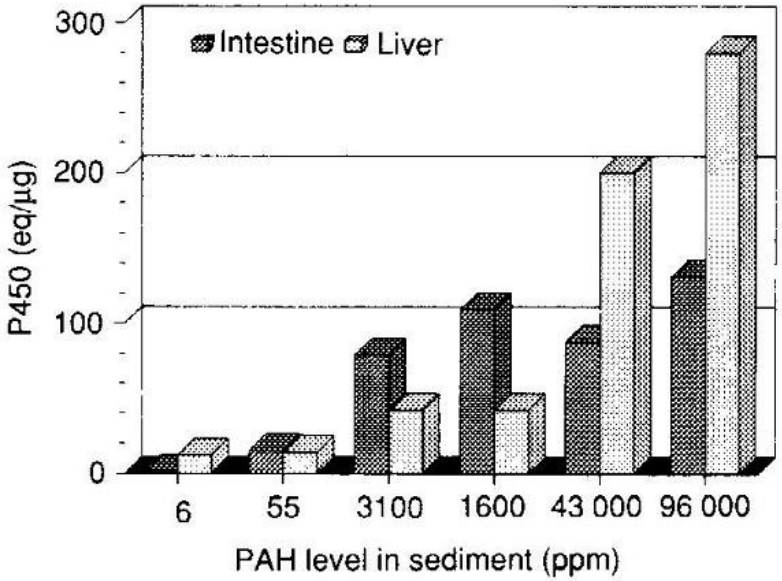


Figure 5.6 Relationship of sediment concentration of PAHs to EROD activity in liver and intestine of spot. After Van Veld *et al.* (1990).

Locality:
Reference Exposed

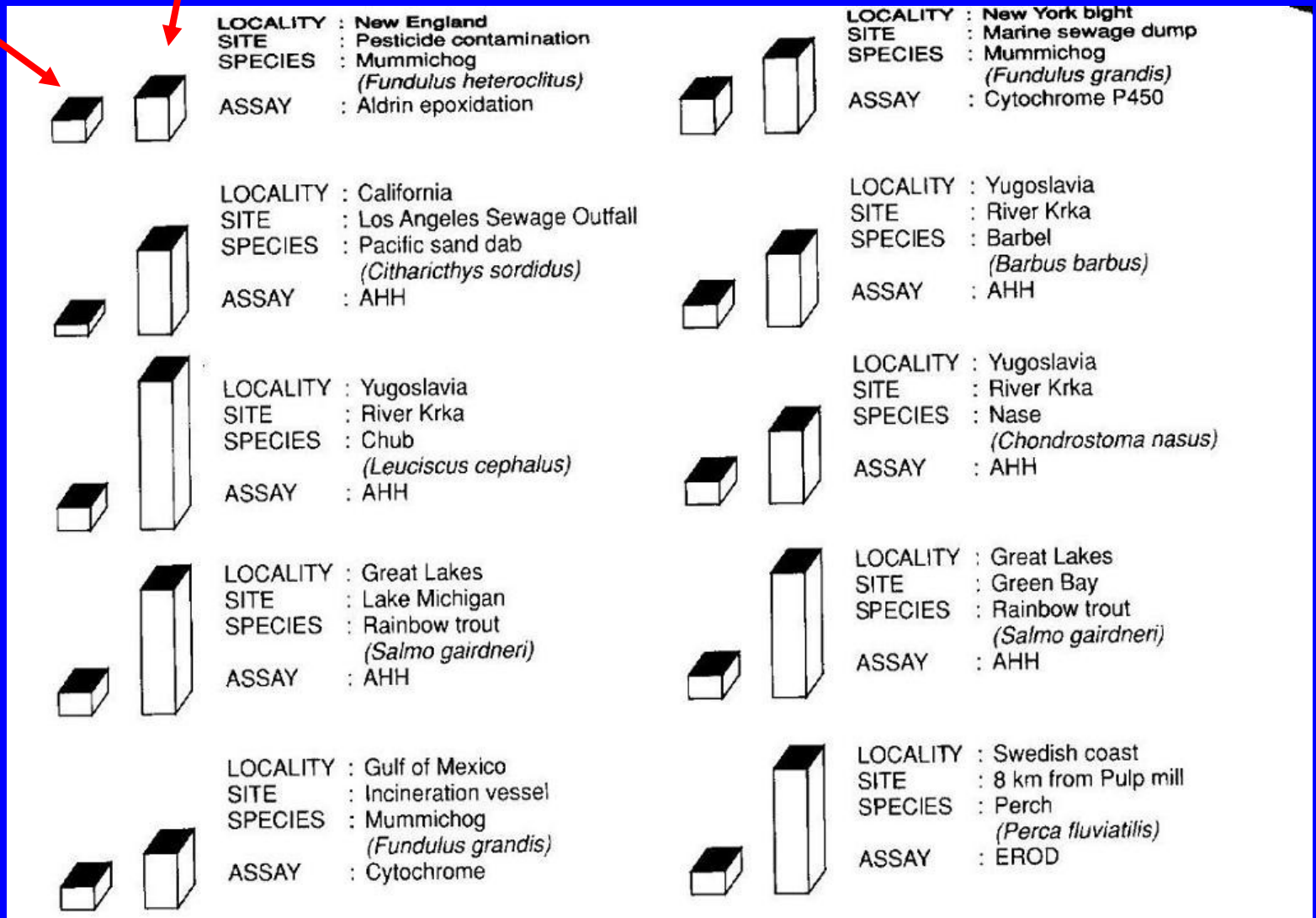
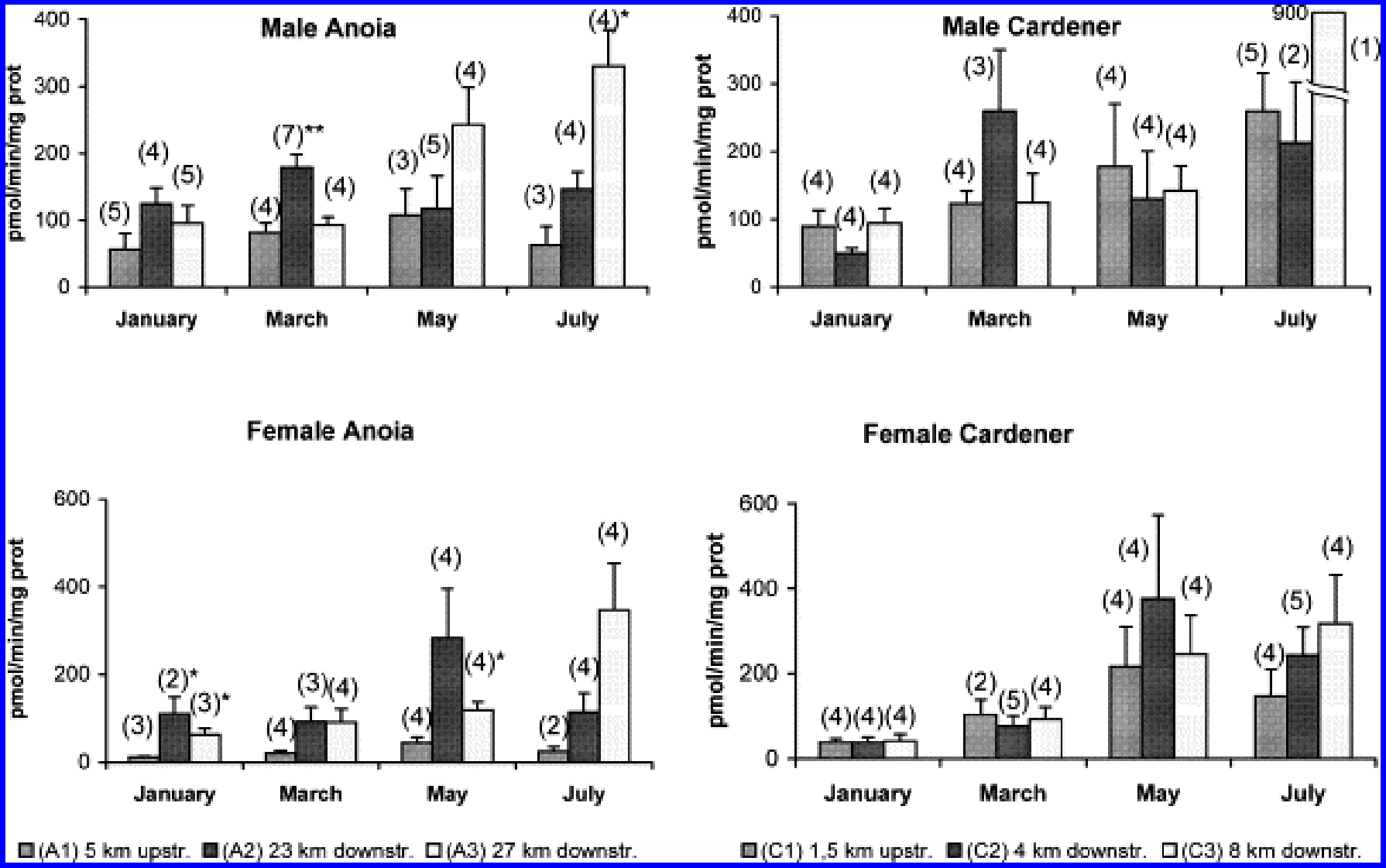


Figure 5.5 MFO changes in fish exposed to organic contamination. The proportion of either enzyme or cytochrome P450 levels detected at reference (short towers) and experimental sites (long towers) is presented in schematic form. All differences between reference and experimental sites were statistically 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 are 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 are SPECIES – SPECIFIC
& relative activity decreases with body size

Related to the general metabolism rate

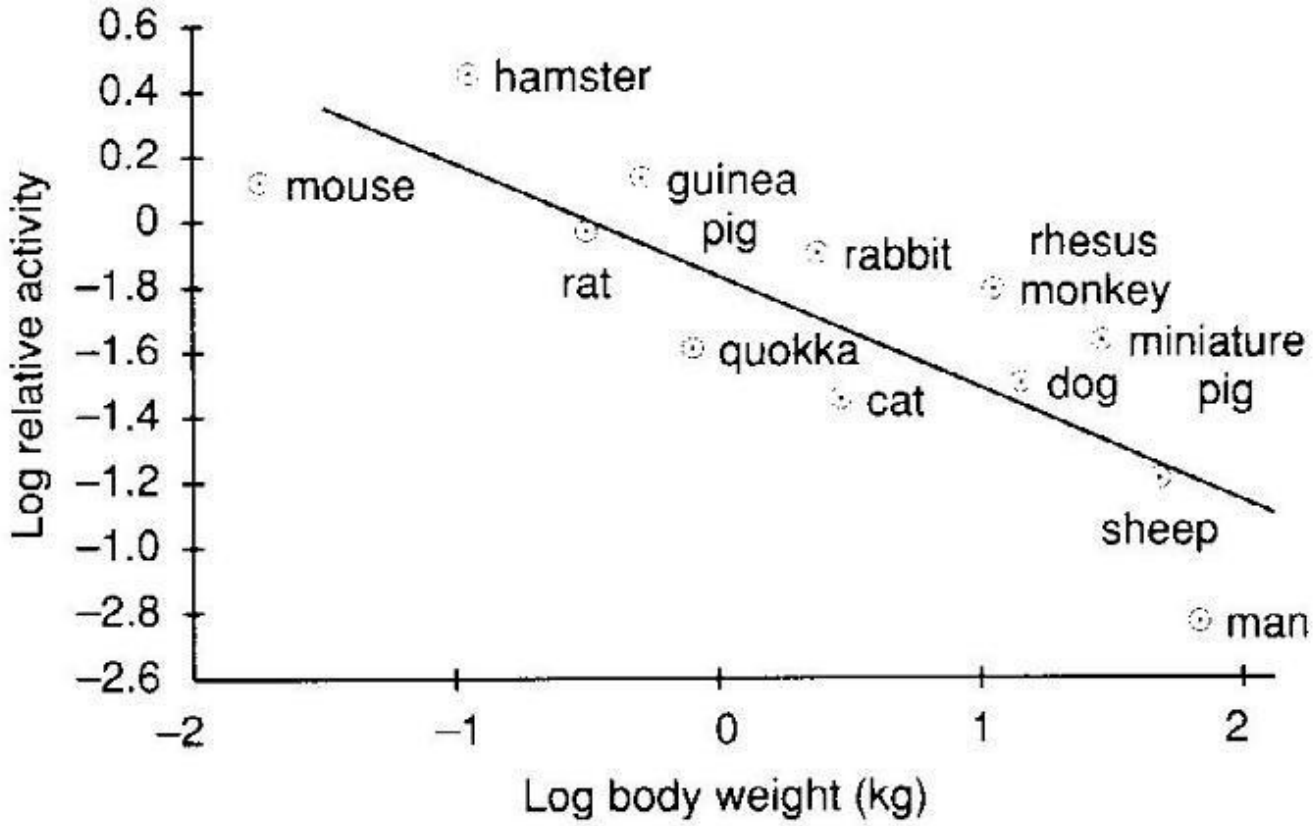


Figure 5.3 Relationship of body weight to MFO activity in mammals. Walker (1978 and 1980).

Phase II conjugation enzymes - GSTs

GSTs

- soluble and membrane (ER) variants
- activities in cytoplasm or microsomes

Methods

Substrates:

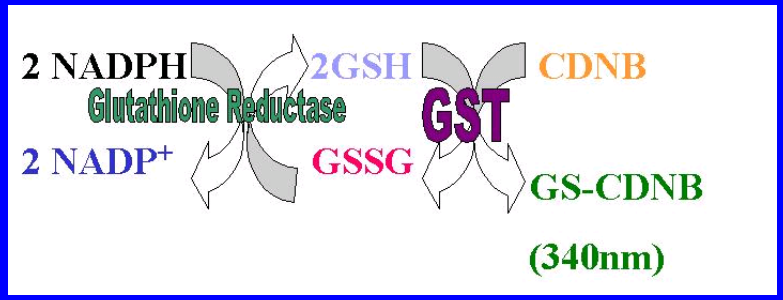
reduced GSH

+ **thiol selective probe (CDNB)**

GST

GSH + CDNB -> GS-CDNB

yellow product, kinetic or endpoint determination



Kinetic assessment

stress -> Induction of GSTs

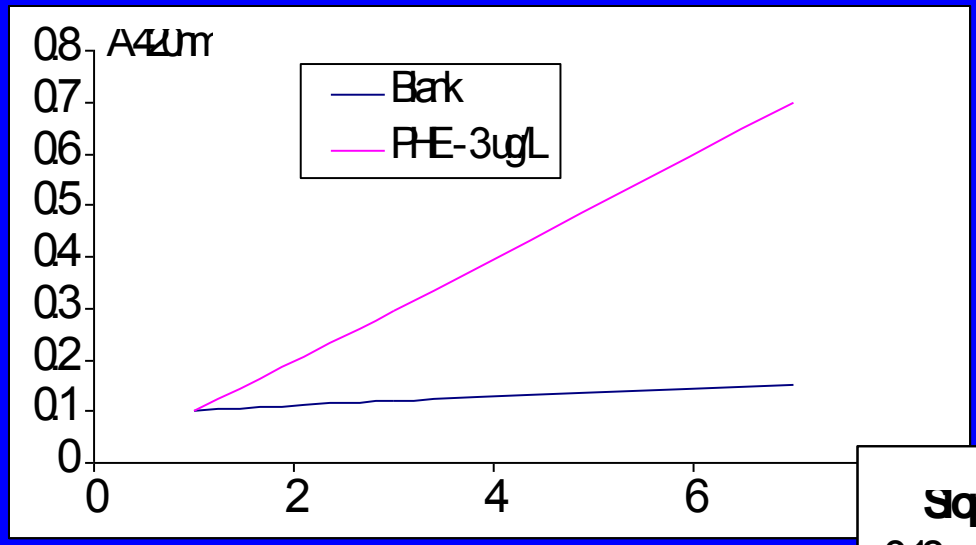
faster reaction -> slope of kinetic increase

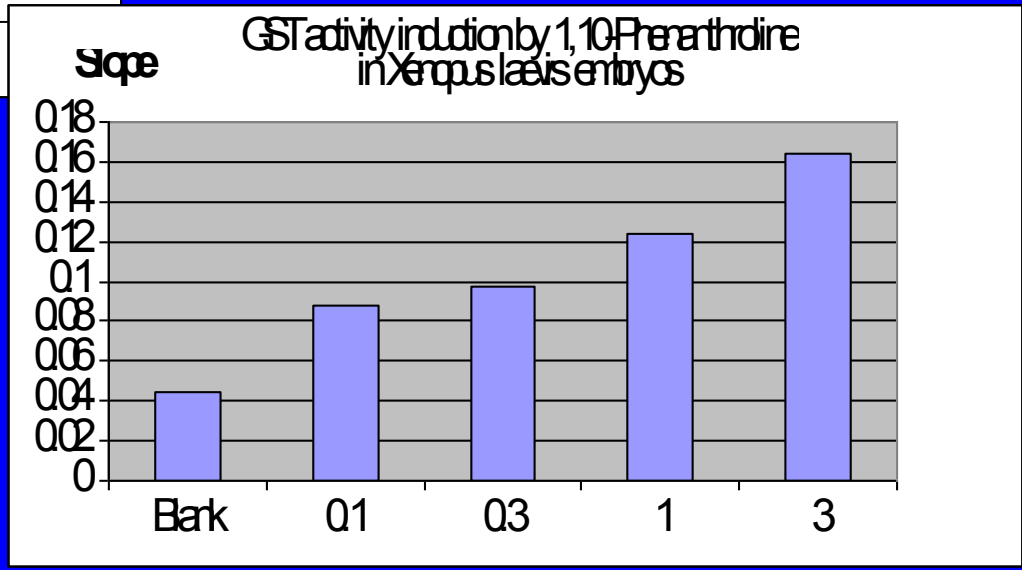
GST activity - example

Kinetic assessment of GSTs

stress -> Induction of GSTs

faster reaction -> kinetic slope increases





Protein levels (synthesis) biomarkers

PROTEIN SYNTHESIS

Protein determination

- amount (concentration)

- activity (see enzymatic assays)

Amount quantification

- mRNA levels (*in vitro assays)*
- protein levels
  - electrophoresis and Western-(immuno)blotting
  - ELISA techniques

Examples

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

metalothioneins

Vitellogenin(-like) Vtg proteins in male

Aromatase

Heat Shock Proteins (hsp)

Stress = synthesis of new proteins

~ equilibrium and homeostasis buffering

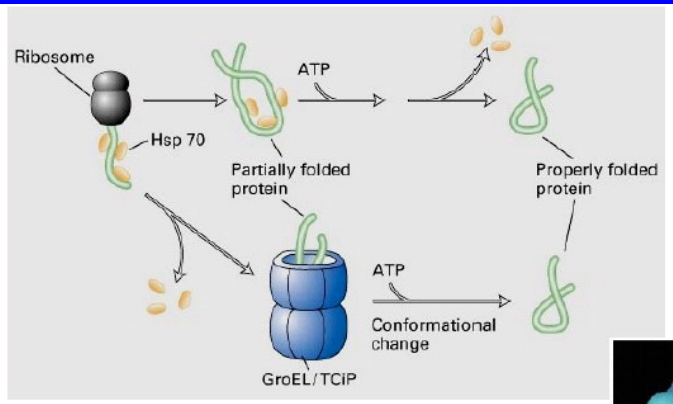
- temperature (cold / heat) – cryo-preservation
- salinity & metals – ion buffering
- organic xenobiotics – detoxication

New proteins must be folded

(3D-structure) by „CHAPERONES“

- hsp90, hsp60, hsp 70

(~ 60-90 kD molecular weight kD)



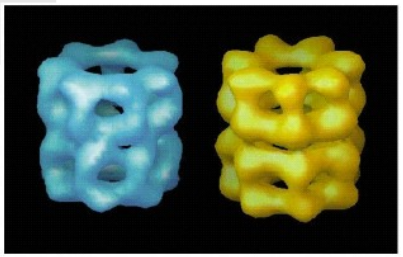


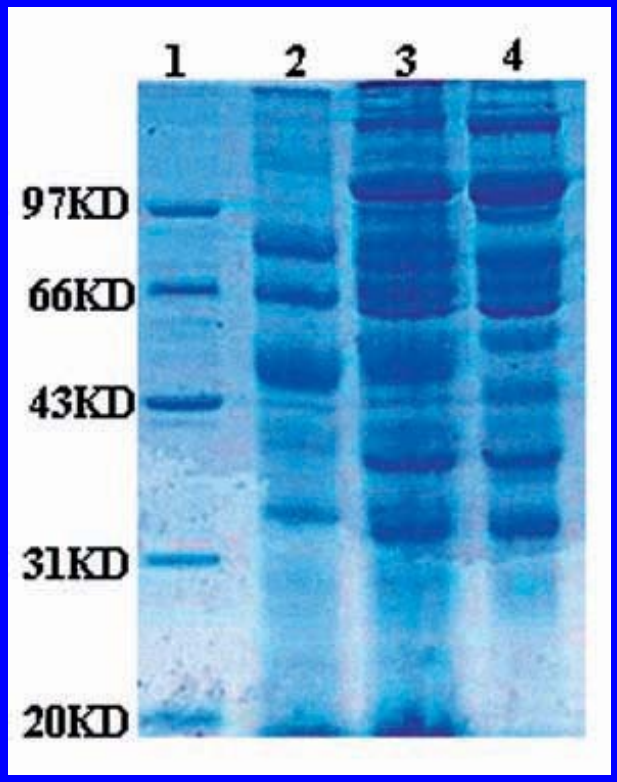
Figure 3-15

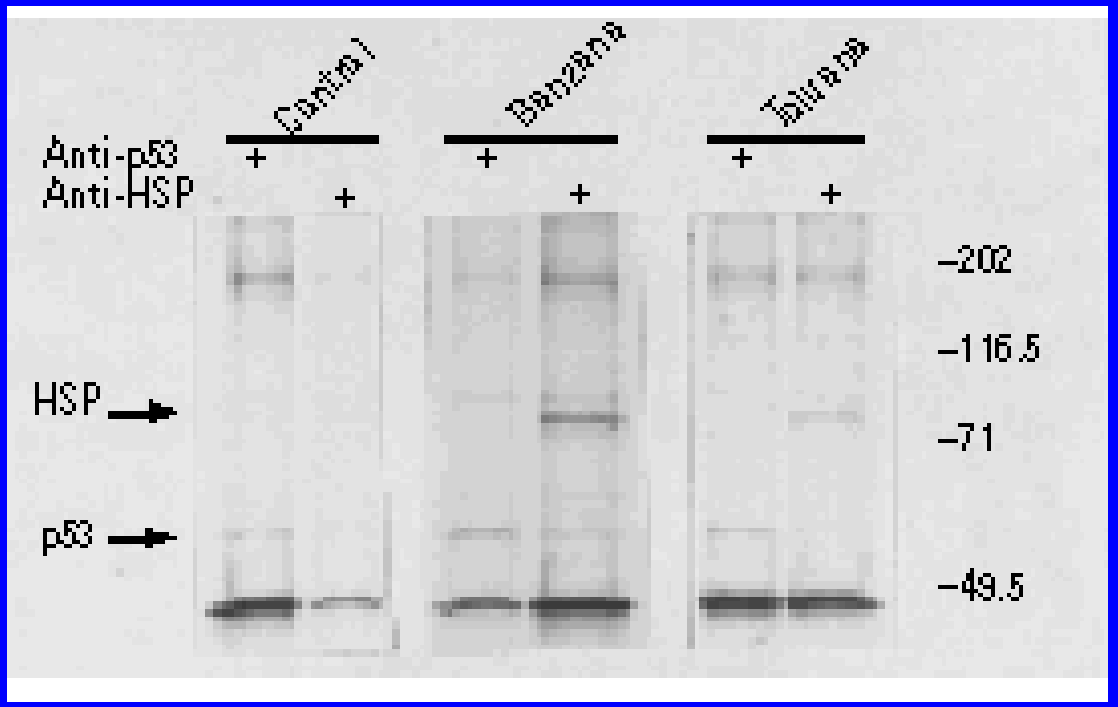
HSP determination - example

HSP = GENERAL STRESS biomarker, non-specific

- phylogenetically conserved (similar sequences in „all“ 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

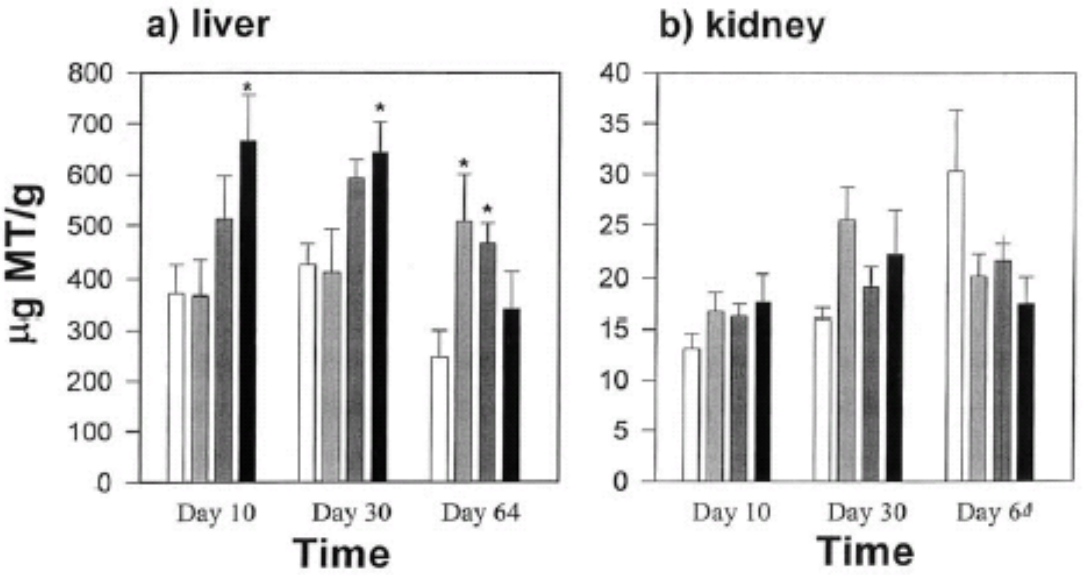


Fig. 2. Metallothionein (MT) concentrations in the (a) livers and (b) kidneys of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (± S.E.). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$).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

Protein biomarkers of estrogenicity

ERs (transcription factors) control number of target genes

Target genes = biomarkers of estrogenicity

- Vitellogenin
- Aromatase - CYP19A

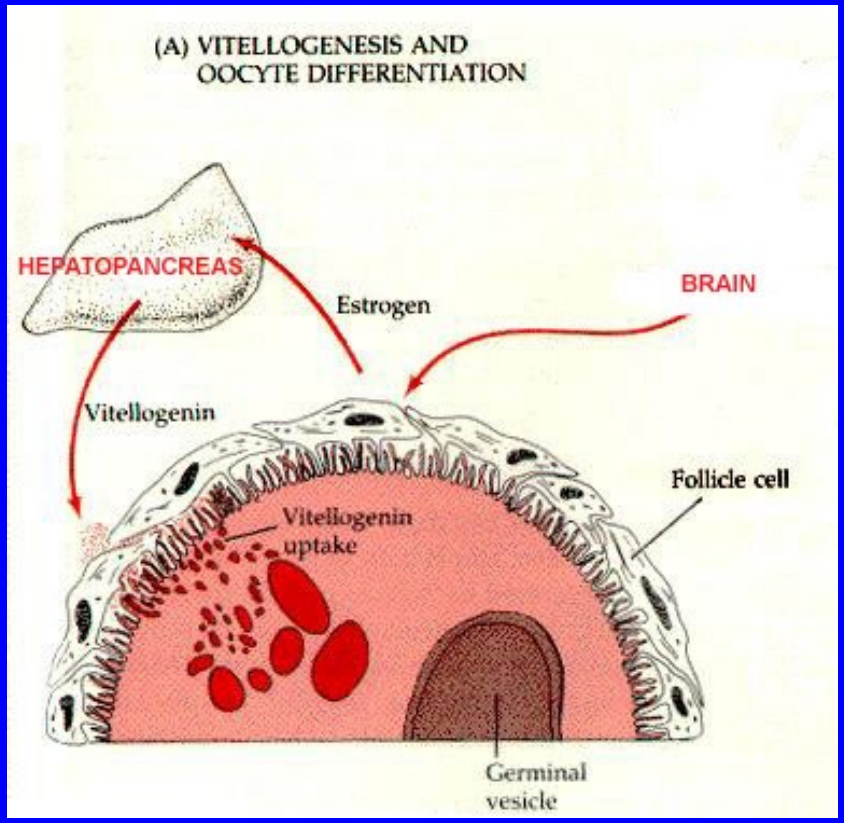
Vitellogenin

Vtg

- precursor of yolk proteins, phospho-protein
  -> egg formations (females) at oviparous animals

- synthesised in liver and distributed via blood (haemolymph)

: xenoestrogens & other endocrine disruptors
  -> increased levels or early production in FEMALES
  -> production in MALES



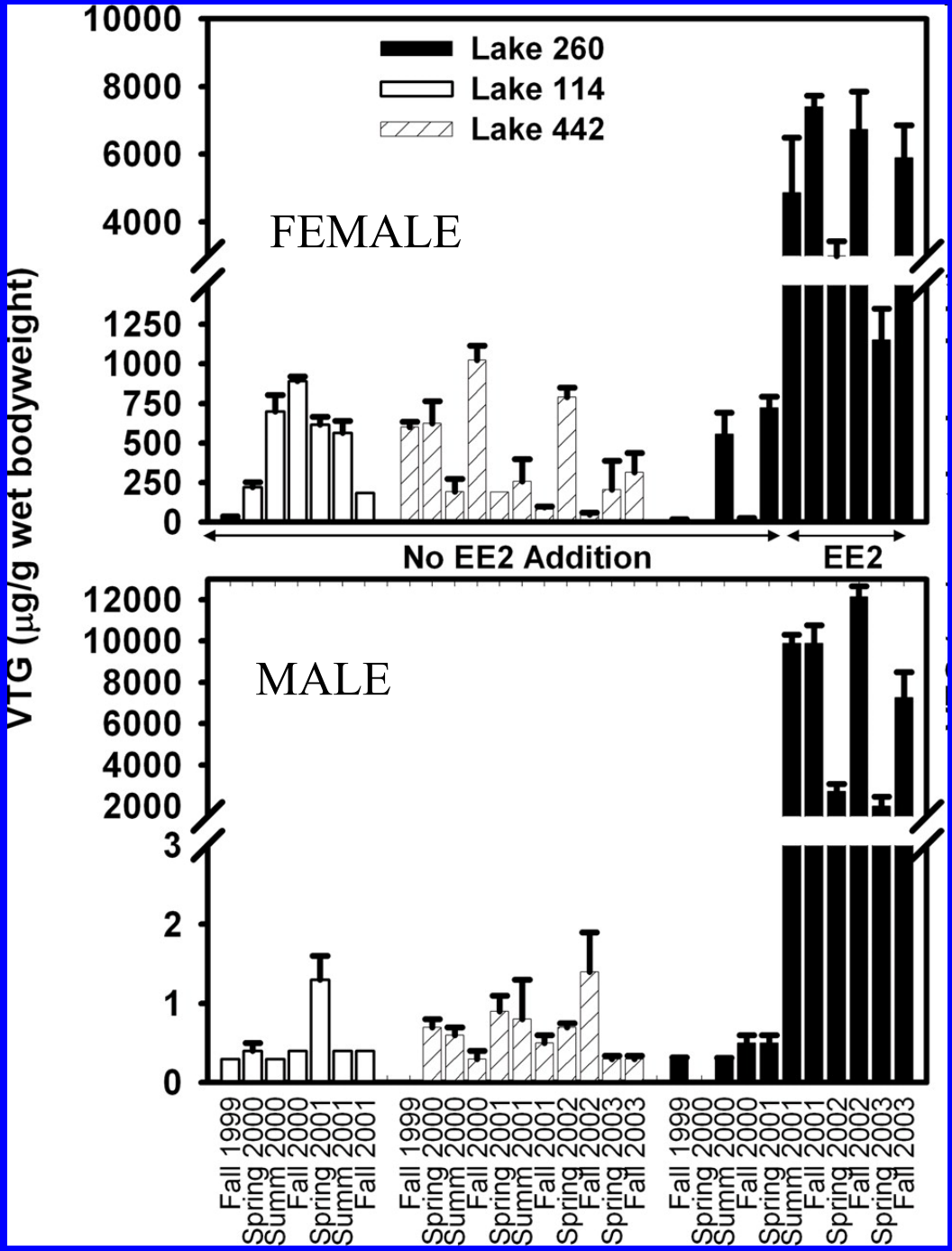
Vitellogenin

VTG Determination

1) ELISA (exposed organisms - F/M, in vitro
- in vivo - exposed organisms (*biomarker in vivo*)
- in vitro production in hepatocytes exposed to effluents
(marker of estrogen-like presence
(-) specific Antibodies necessary for each species (low crossreactivity)

2) „Vitelin-like proteins“
- total amount of „alkali-labile“ phosphate in haemolymph (mussels)
- alkaline extraction of P from sample & 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).

Vitelin-like proteins in mussels

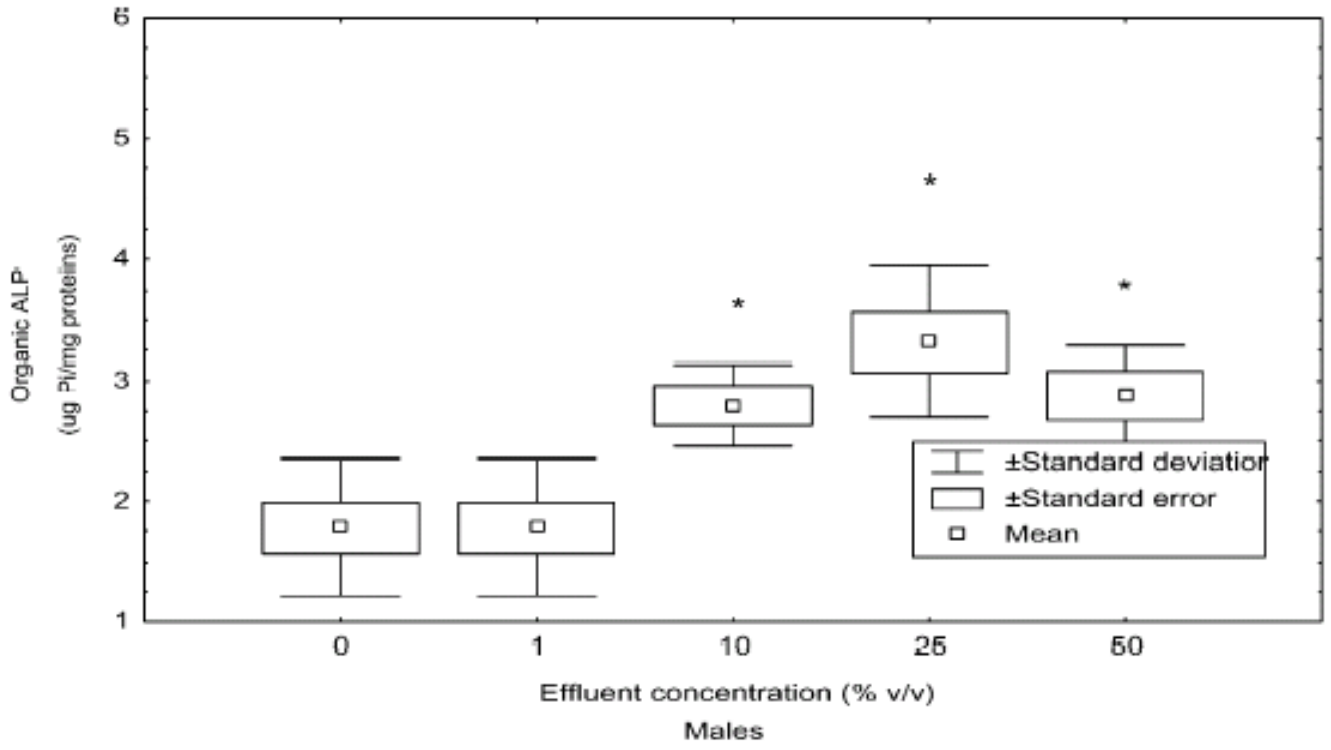
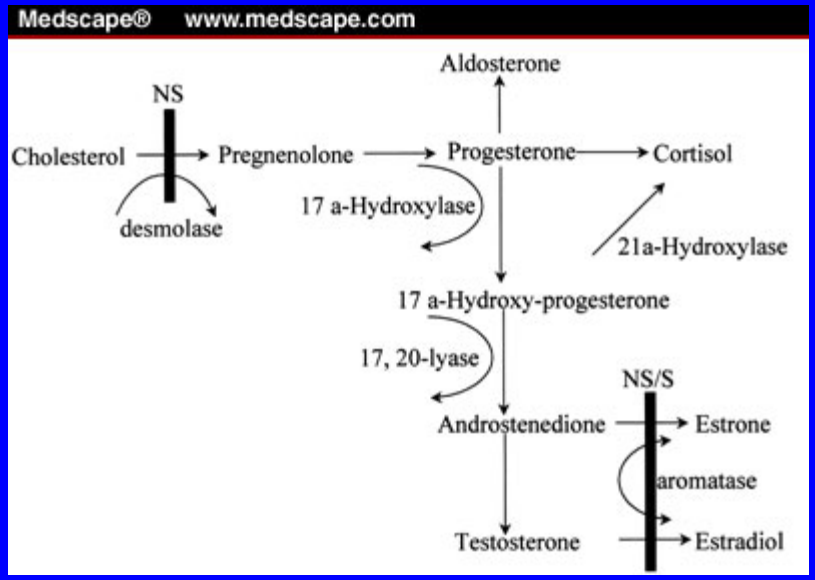


Fig. 4. Induction of Vg by exposure to a municipal effluent. Mussels were exposed for 96 h to a municipal effluent at 15°C. They were then collected for Vg and sex determinations. The asterisk (*) indicates significant difference at $P < 0.05$.

Aromatase (CYP19A)

Aromatase
- inducible by estrogens
- single enzymatic step
androgens → estrogens

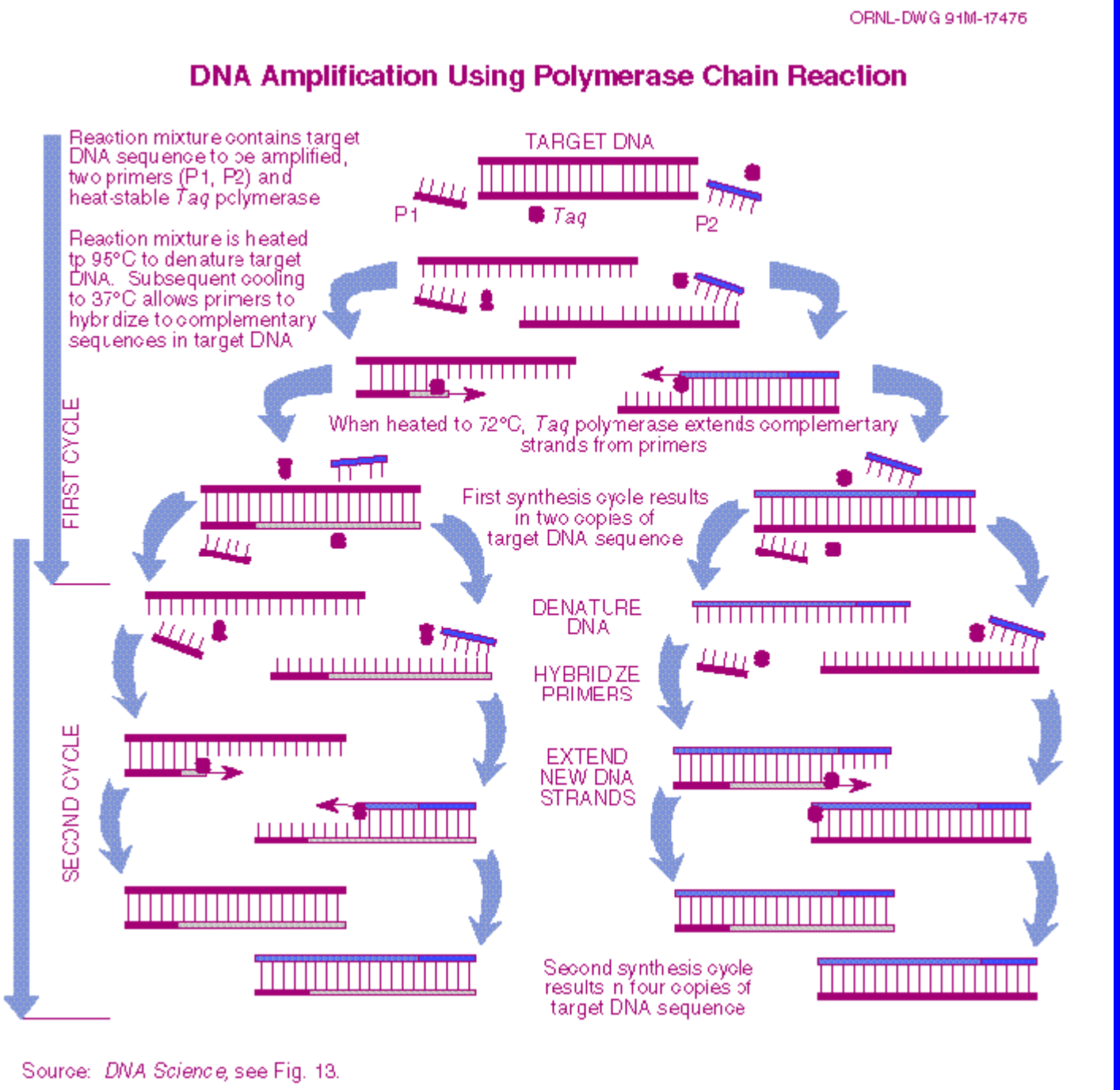


Experimental assessment
(in reseach and practice)

- PCR / Quantitative-Real-Time-PCR

- GM-organisms (zebrafish)
(reporter gene – GFP – under the control of aromatase promoter)

PCR principle

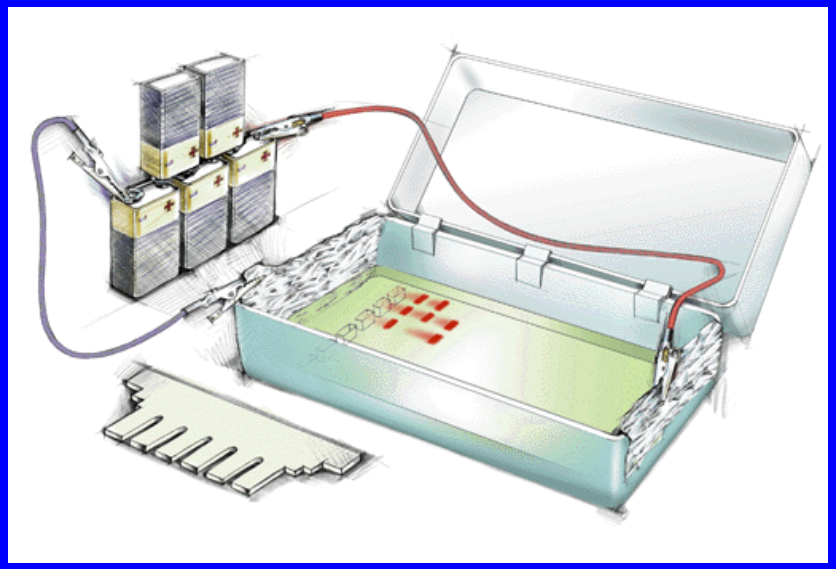


Source: *DNA Science*, see Fig. 13.

Visualization of the PCR product

1) **Electrophoresis (qualitative)**

Dyes – e.g. **ethidium bromide**



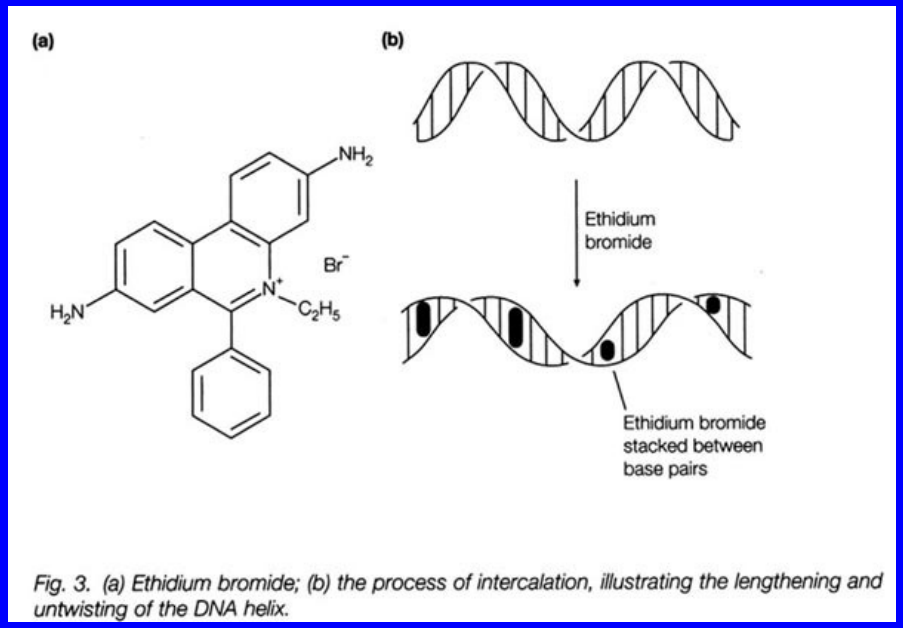
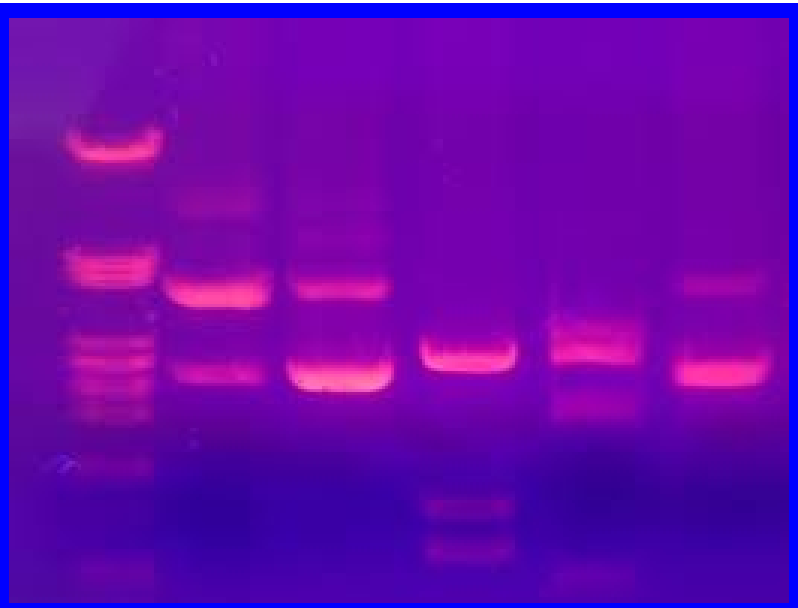


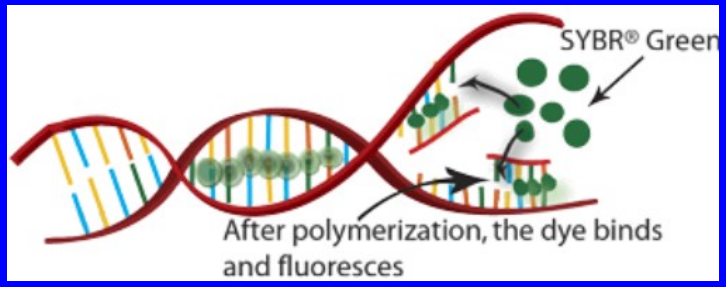
Fig. 3. (a) Ethidium bromide; (b) the process of intercalation, illustrating the lengthening and untwisting of the DNA helix.

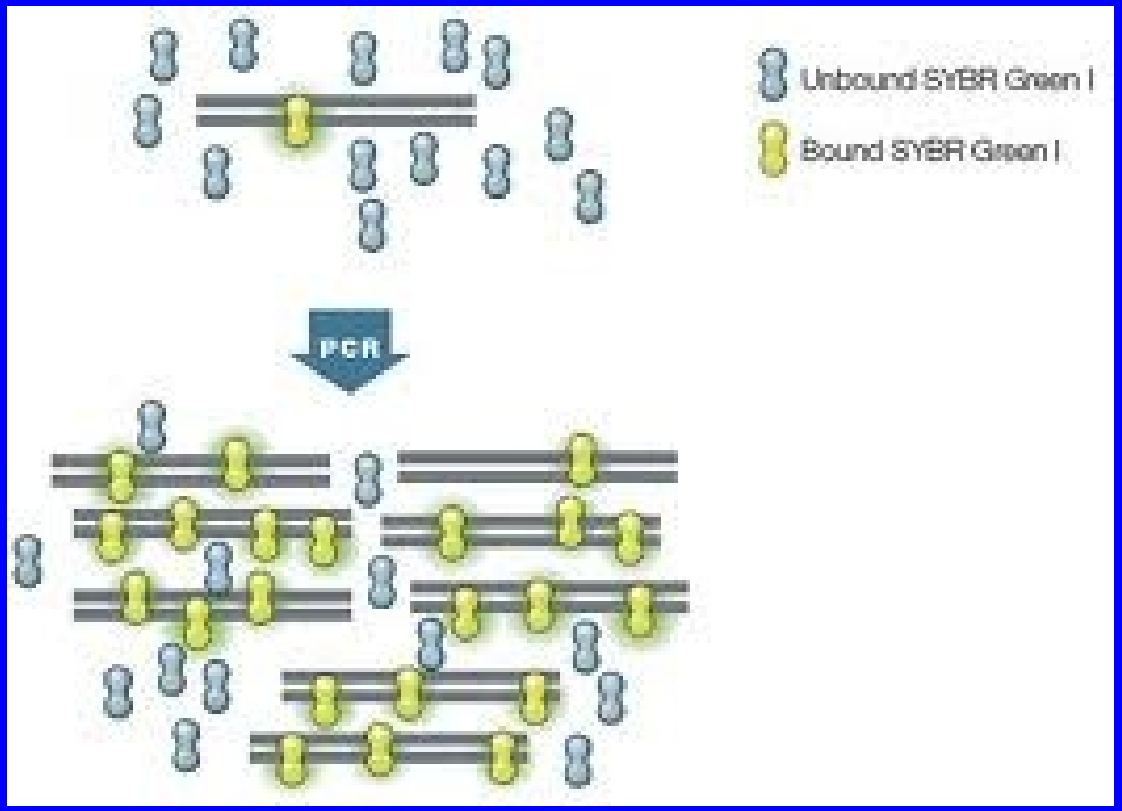


Visualization of the PCR product

2a) Real-time (quantitative) SYBR GREEN

- (more DNA synthesized,
  more fluorescent dye incorporated)



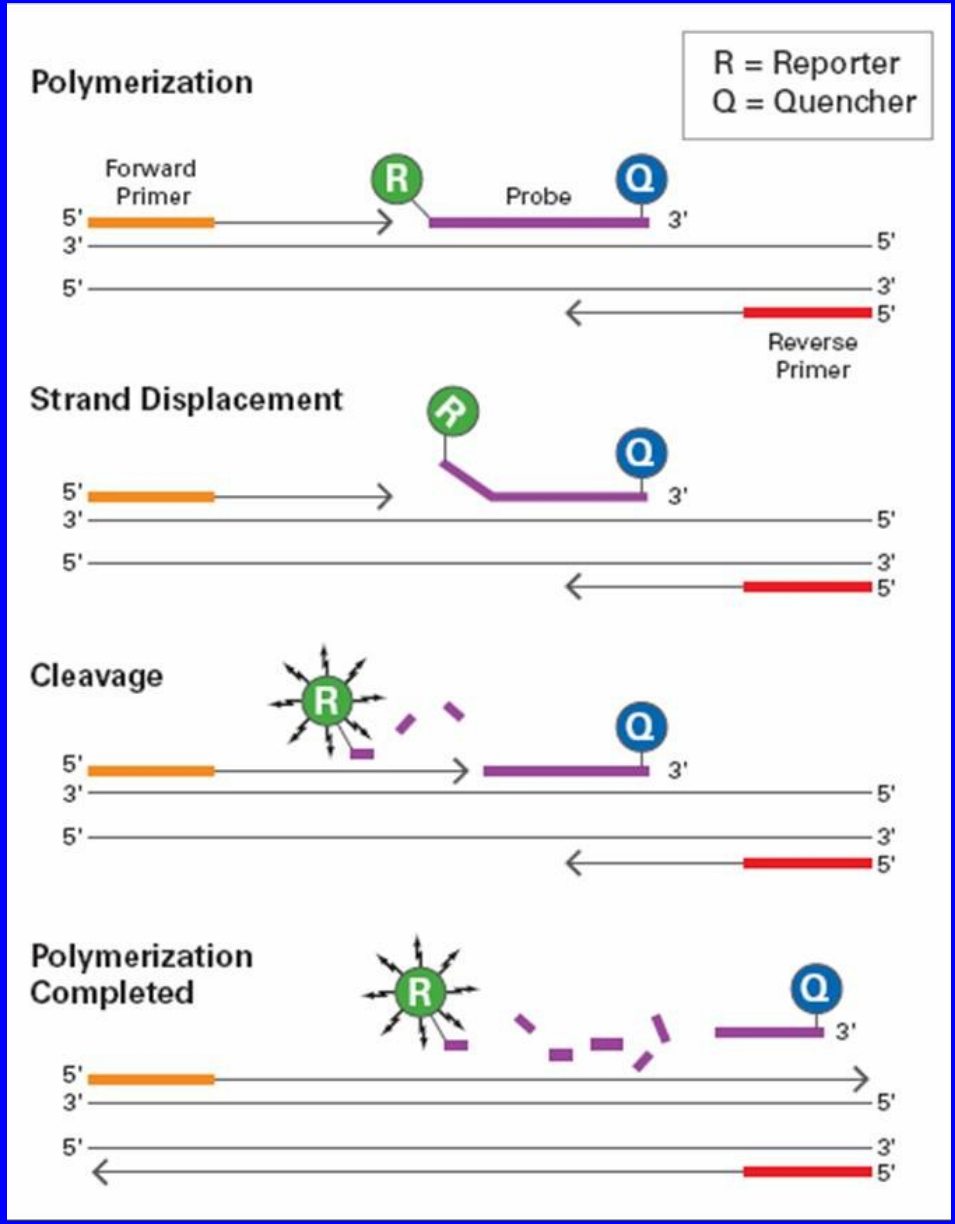


Visualization of the PCR product

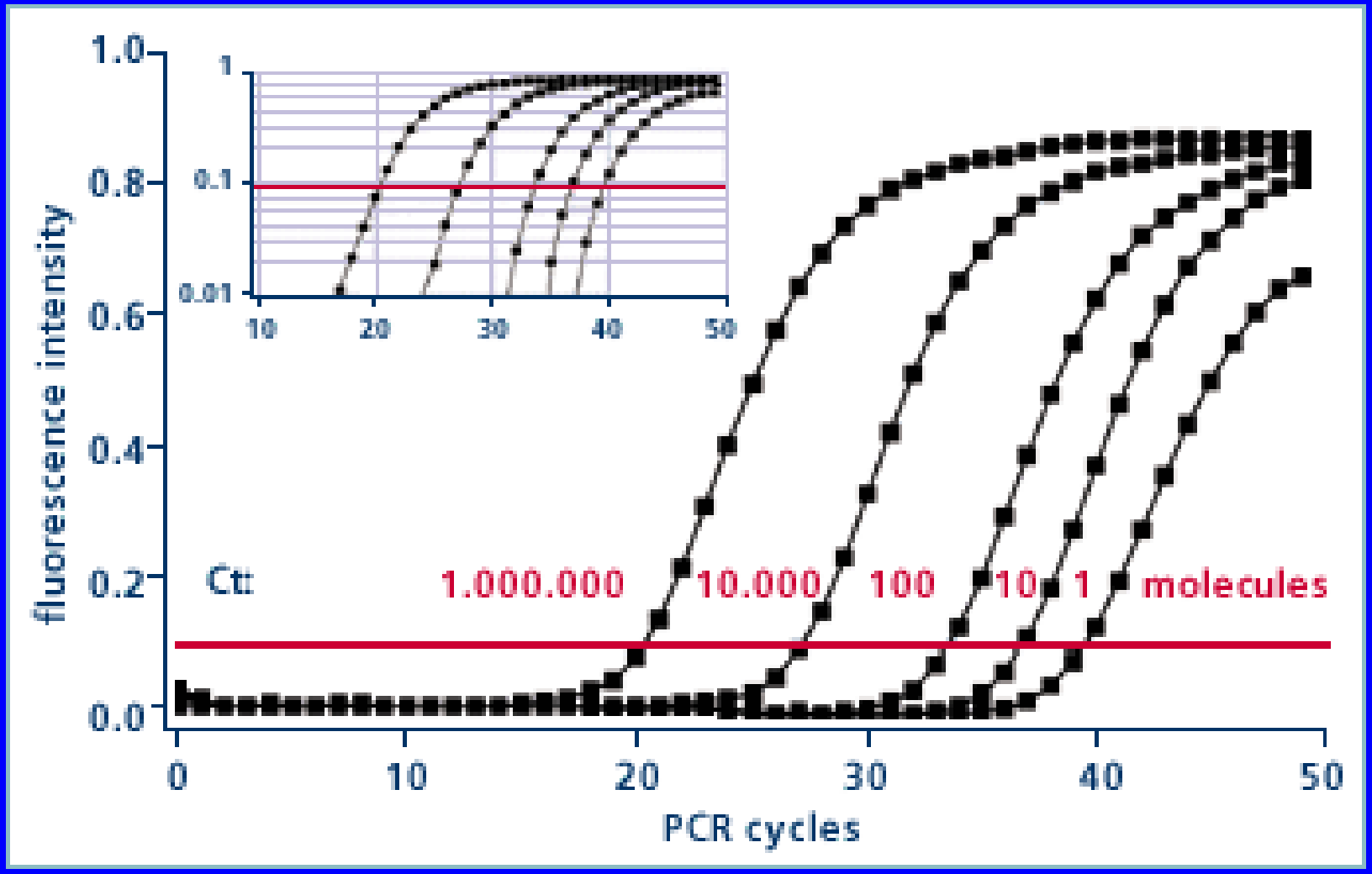
2b) Real-time (quantitative)

- **TaqMan probes**

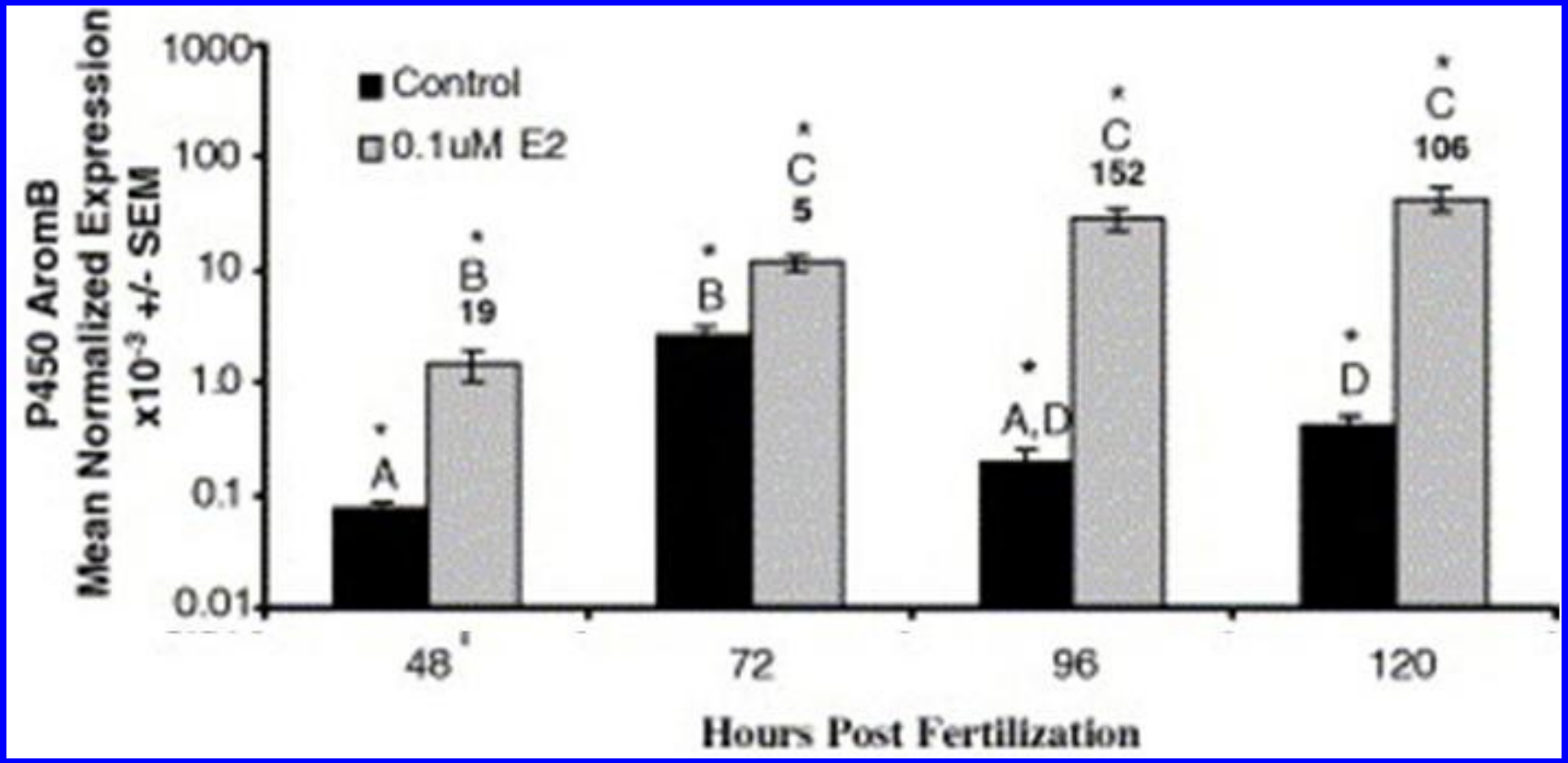
(more DNA replications
more fluorescent dye released)



Visualization of the PCR product

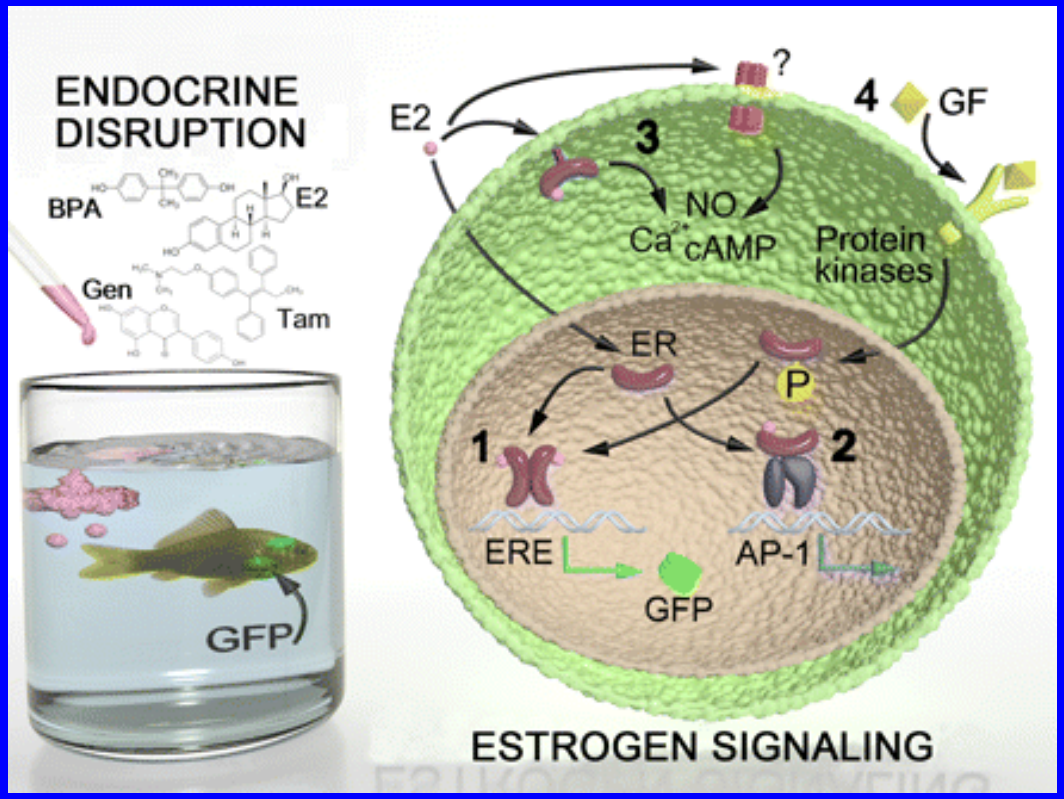


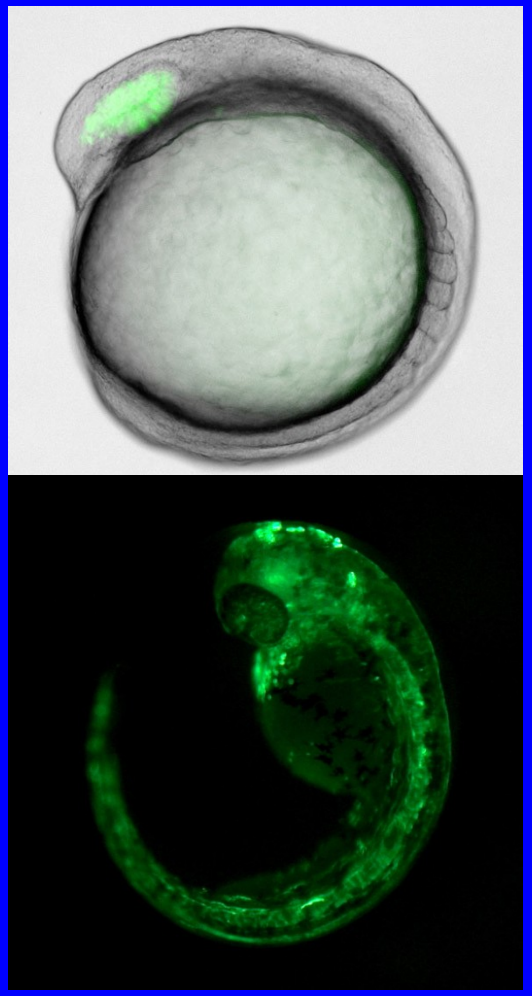
QPCR determination of the Aromatase gene expression in Zebrafish



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

GFP-reporter for estrogens (zebrafish embryo)





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

Biomarkers of oxidative stress

Oxidative stress markers

Several parameters respond to oxidative stress

: enzymes (GPx, GR, GSTs)

- *enzymatic activities (see elsewhere)*

: antioxidants (**GSH**, vit E)

: markers of oxidative damage

- **MDA**,
- *8OH-dG (see DNA damage)*

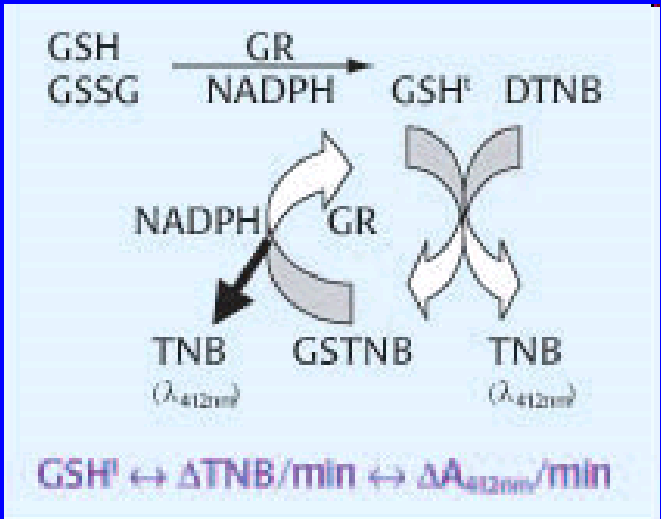
Oxidative stress markers

GSH determination

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

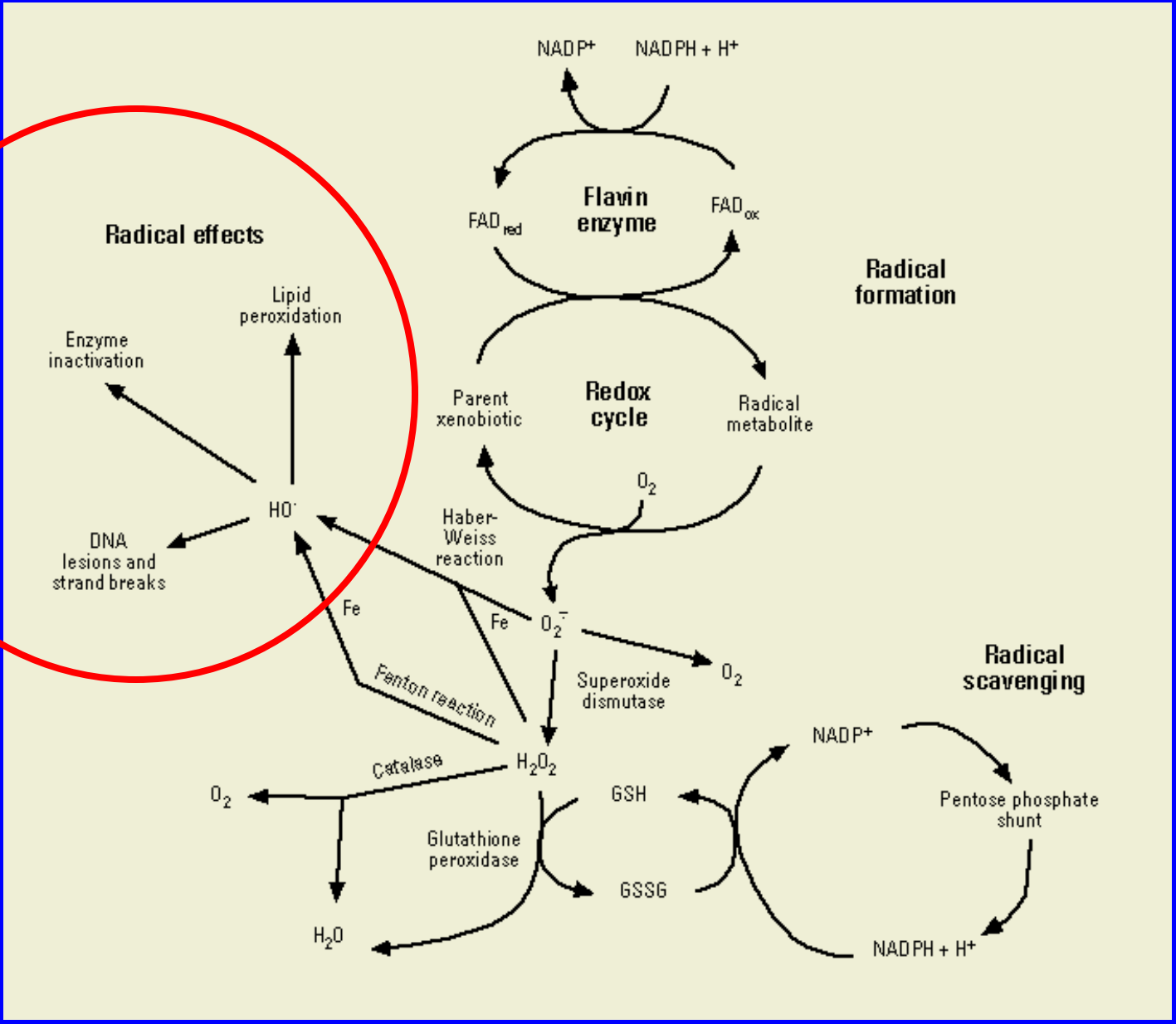
Total glutathione = reduced GSH + oxidized GSSG

GSH + **Ellman´s reagent (DTNB)** -> Reduced GSH
GSH + Glut.Reductase + **DTNB** -> Total GSH



Total – Reduced = Oxidized

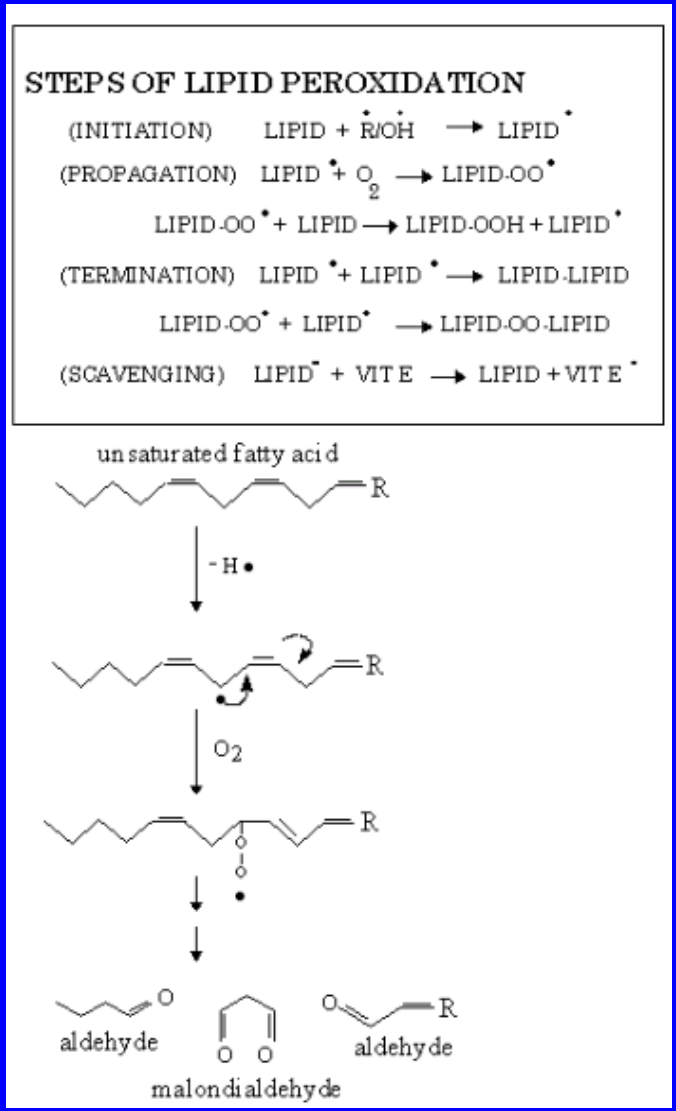
Markers of oxidative DAMAGE



Lipid peroxidation -> Malondialdehyde (MDA)

MDA – malondialdehyde

product of Lipid peroxidation



Lipid peroxidation
-> Malondialdehyde (MDA)

MDA – formed from oxidized membrane phospholipids

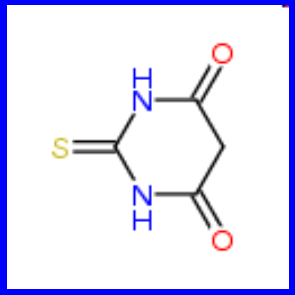
: determination:

- HPLC
- TBARS method

TBARS – ThioBarbituric Acid Reactive Species

: less specific than HPLC (+/- aldehydes)

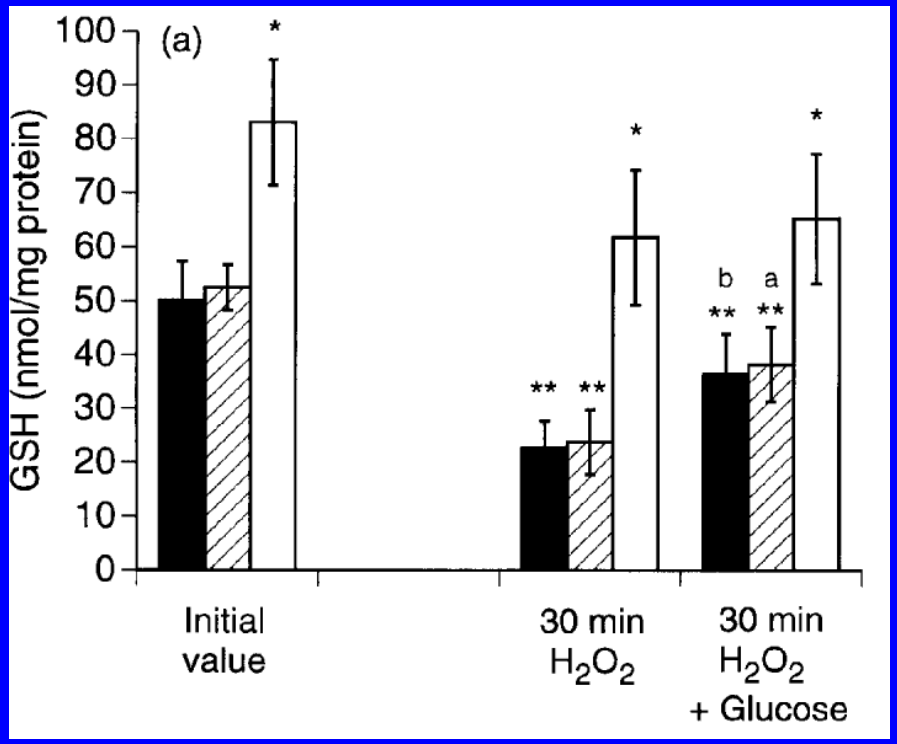
: easy determination (spectrophotometry)



<u>Method:</u>

1) sample extract *(with MDA)*
2) add TBA
3) boil (cca 30´ / 90°C)
   => formation of <u>red/violet coloured product</u>
4) determination by spectrophotometry (A 540 nm)

MDA modulation - examples



Effects of antioxidants in young/old on oxidative damage (MDA)