Biomarkers

Biomarkers

- markers in biological systems with a sufficently long half-life which allow location *where* in the biological system change occur and *to quantify* the change.

Toxicology – present status:

- identification of markers of long-term risks
  : human toxicology – carcinogenesis
  : ecotoxicology – early markers of toxic effects

Biomarkers - summary

Biomarker:

change which occurs as response to "stressors" (xenobiotics, disease, temperature...) which extend the adaptive response beyond the normal range

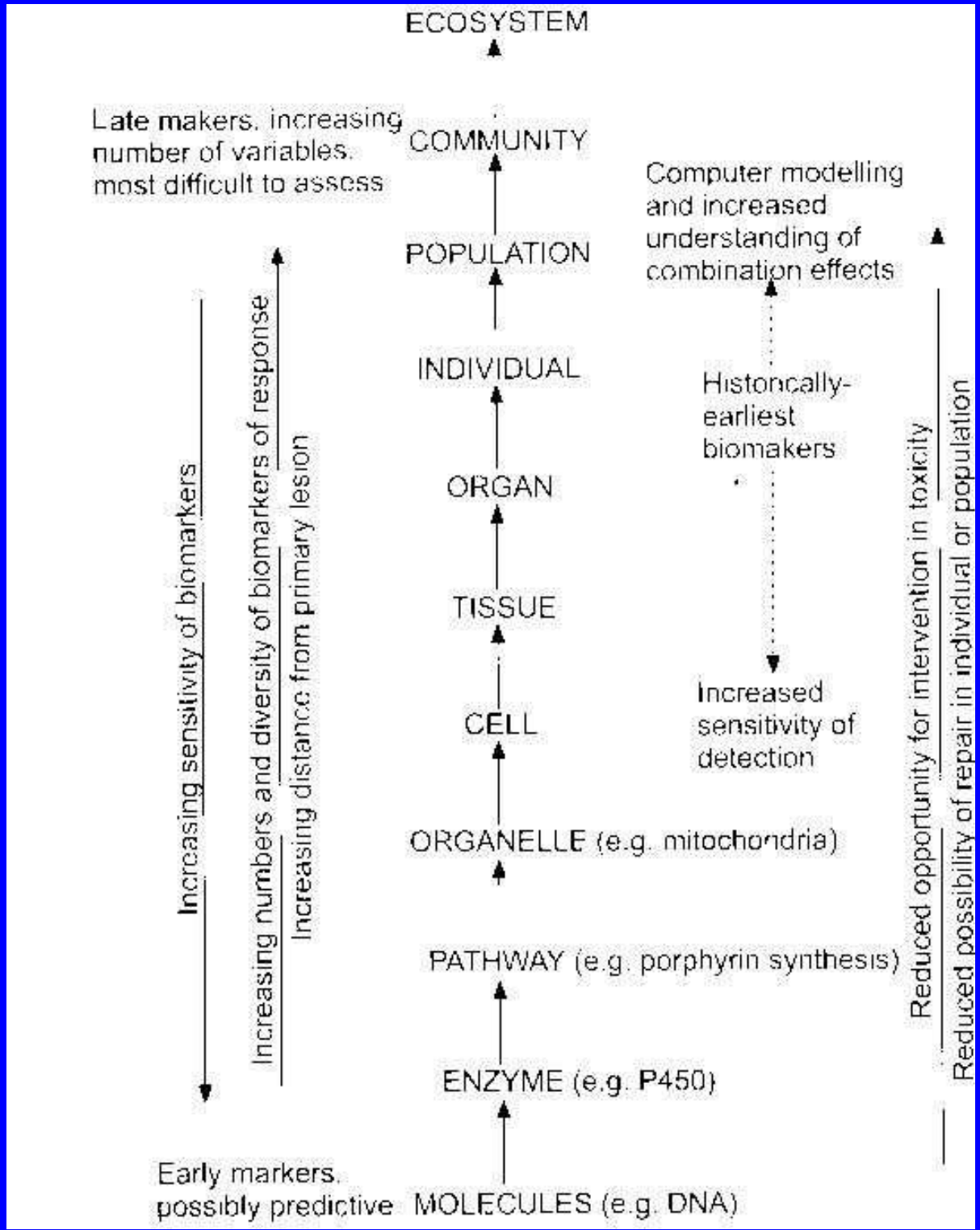
In vivo biomarkers:

changes measured in stressed animals ("classical biomarkers")

In vitro biomarkers

in vitro assessment for characterization of xenobiotic potencies to induce specific biological activity *(genotoxicity, estrogenicity, dioxin-like activity, tumor promotion ...)*

Biomarkers at different levels of biological organisation



Biomarkers - classification

Categorization according to US Nat. Academy of Science

- Biomarkers of exposure
- Biomarkers of response or effect
- Biomarkers of susceptibility

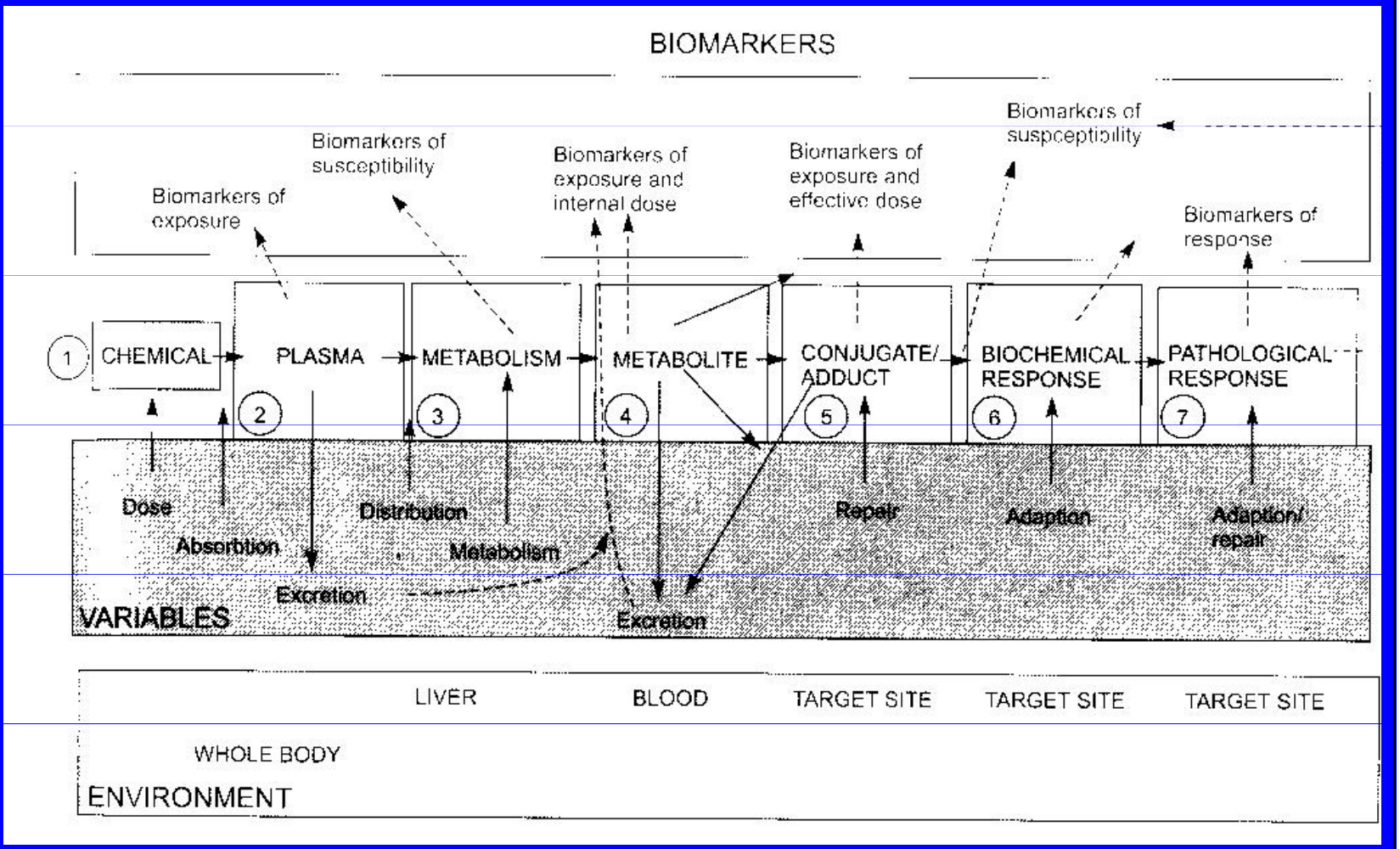
Continuum exists

adducts with DNA *? response / ? exposure*

Biomarkers & sampling

invasive / non-invasive

Biomarkers
- Paracetamol



(1) paracetamol

(2) parent compound measurement - *exposure*

(3) activation to reactive metabolite (N-ac-p-benzoquinone, NAPQI) by CYPs; reaction with GSH / measurement – levels of CYPs; levels of GSH – *susceptibility*)

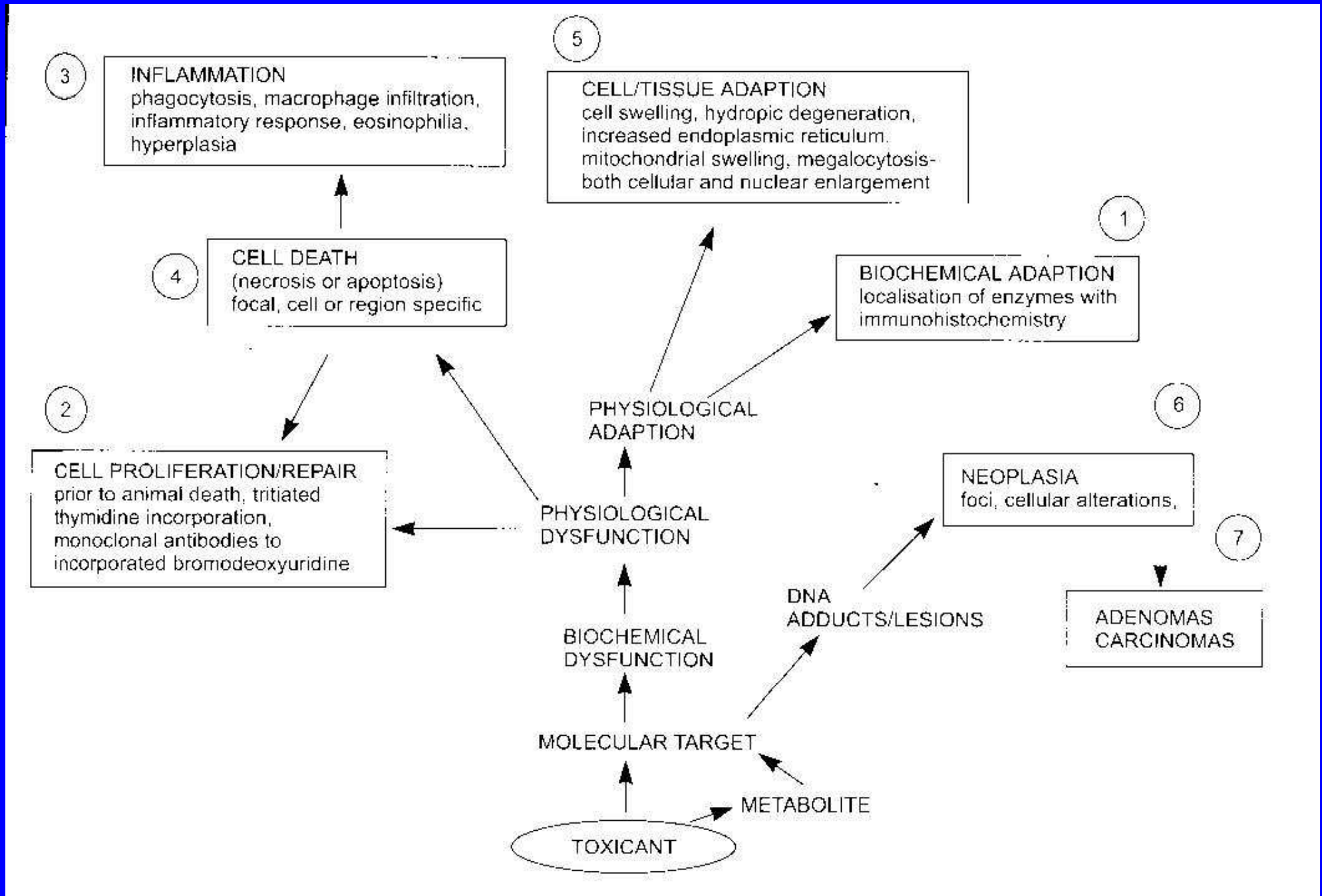
(4) GSH-NAPQI conjugate – *exposure, susceptibility*

(5) NAPQI-protein adducts -> toxicity: *exposure, effective dose*

(6) adaptations: GSH depletion, inhibition of protein synthesis – *biomarkers of response*

(7) protein alkylation -> degeneration of hepatocytes: necrosis -> increase concentrations of bile acids, bilirubin in plasma; start of inflamation in degraded tissue – *response / effect*

Human biomarkers – example



Human biomarkers – example

Table 1 Examples of different biomarkers illustrated with specific examples and examples of the stressor which may result in the biomarker changes

Type of biomarker	Biomarker	Specific example	Stressor
Exposure	DNA adducts	Styrene oxide-O^6 guanine	Styrene exposure
	Protein adduct	N^7-Guanyl-aflatoxin B_1	Dietary aflatoxin
	DNA fragments	7,8-Dihydro-8-oxoguanine	Reactive oxygen species
Exposure and effect (response)	Protein adducts	Carboxyhaemoglobin	CO inhalation
	Enzyme inhibition	Acetylcholinesterase inhibition	Organophosphates
	Urinary metabolites	Mercapturic acids	Buta-1,3 diene, allyl chloride
Effect (response)	Serum/plasma enzymes	AST (aspartate aminotransferase)	Xenobiotics causing necrosis
		LDH (lactate dehydrogenase)	Xenobiotics causing necrosis
		ALT (alanine aminotransferase)	Hepatotoxic compounds
	Serum/plasma biochemistry	ALP (alkaline phosphatase)	Bile duct toxins
		CK or CPK (creatine kinase)	Heart/muscle toxins
		Urea (changes)	Hepatotoxic and nephrotoxic compounds
		Protein (reduced, e.g. albumin)	Hepatotoxic compounds
		Bilirubin	Liver injury
	Clotting time	Prothrombin	Warfarin (rodenticide)
	Urinary metabolites	Glucose, raised creatinine, GSH conjugates	Pancreatic abnormalities, kidney damage
	Raised antioxidant levels	Liver glutathione	Reactive oxygen species
	Enzyme induction	P450 induction	Polycyclic aromatic hydrocarbons
	Stress proteins	hsp 60, hsp 70, hsp90	Cadmium, heat
	Protective proteins	Metallothionein	Heavy metals, e.g. cadmium
		Antibodies, e.g. IgG	Antigens
	Allergic response	Dermatitis	Nickel
	Histology	Chromosomal aberrations, micronuclei	Genotoxic agents
	Clinical observations	Heart rate, temperature, sleeping time	Barbiturates
	Population studies	Breeding patterns, migrations	Climate change
Susceptibility	Phenotype	Acetylator phenotype (*NAT 2*)	–
	Oncogenes	Dominant oncogenes (*ras, mic*)	–
		Recessive suppressor gene (*p52*)	–
	'Cancer' genes	Breast–ovary cancer gene (*BRCA 1*)	–

Specific (selective) *in vivo* biomarkers

- Biomarkers selectively reflecting specific types (mechanisms) of toxicity
- E.g. inhibition of AcCholE :
  exposure = organophosphates; effect = neurotoxicity

+ specific information

- multiple biomarkers must be measured

Non-specific (non-selective) *in vivo* biomarkers

- Biomarkers of general stress
- E.g. induction of Heat Shock Proteins (hsp)

+ general information about stress

- sensitive to many "stressors" (temperature, salinity ...)

In vivo biomarkers

- Non-destructive
  : blood / haemolymph collection & analyses
  : skin, feather, hair ... contamination

- Destructive
  : whole animal -> multiple biomarker evaluation

Non-destructive biomarkers

Table 9.2 Availability of biomarkers in blood

Biomarker	Blood	Tissue of choice	Comment
AChE inhibition	+?	Brain	Effects in blood more transient
Neurotoxic esterases	–	Brain	Enzyme is limited to brain
Biogenic amines	–	Brain	Changes in blood too transient
DNA			
Strand breakage	?	Wide range	Nucleated avian red blood cells are possible
Adduct formation	+	Wide range	Haemoglobin is good substitute for DNA
SCE	+	Wide range	Blood lymphocytes can be used
Degree of methylation	?	Wide range	Nucleated avian red blood cells are possible
MFO	–	Liver	Western blotting technique on leucocytes is possible
Thyroid	+	Thyroid	Circulating levels of T_3 and T_4 are sensitive
Retinols	+	Liver	Advances to use plasma are being made
Porphyrins	+?	Liver	Advances to use plasma are likely
ALAD	+	Blood	Tissue of choice
Enzymes	+	Blood	Tissue of choice
Immunotoxic	–	Lymphatic cells, bone marrow	Limited number of tests available for blood

What kind of biomarkers to measure ?

Do we know possible exposure (toxicant) ?

- specific biomarkers

? estrogenic effects in effluents

? dioxin-like effects, mutagenicity in urban areas

? neurotoxicity (AcChE) in rural areas

Do we expect varying exposure / contamination ?

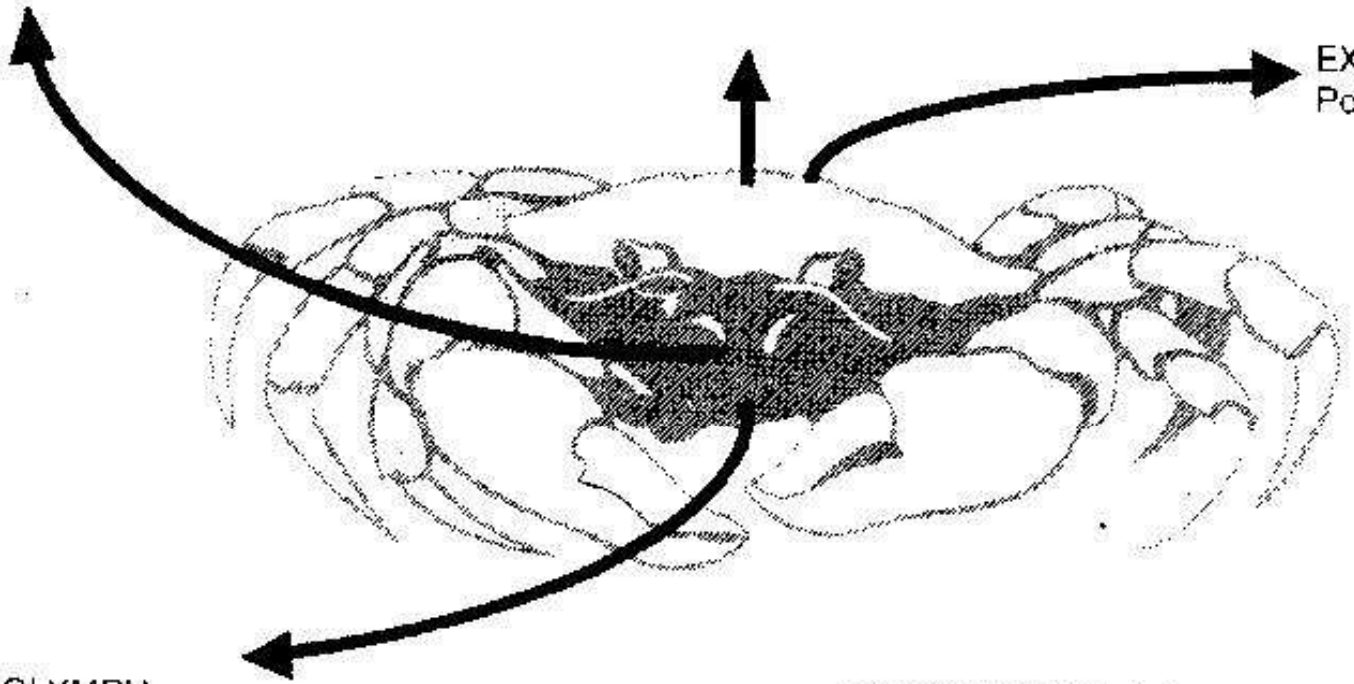
- integrated approach

- non-specific biomarkers (hsp) as predictors of stress level

Multiple biomarker evaluation

GILLS
Benzopyrene mono-oxygenase activity
NADH ferricyanide reductase activity
Micronuclei (mutagenicity)
total proteins

HEPATOPANCREAS
Benzopyrene mono-oxygenase activity
Ethoxyresorufin-O-deethylase
NADPH cytochrome c reductase
NADH cytochrome c reductase
SDS-PAGE for P450
Alkaline unwinding assay (DNA damage)
Porphyrins
Total proteins



EXCRETA
Porphyrins

HAEMOLYMPH
Acetylcholinesterase activity
Butycholinesterase activity
Micronuclei
Total proteins

Hepatopancreas and gills as % of body weight

Biomarkers & Exposure

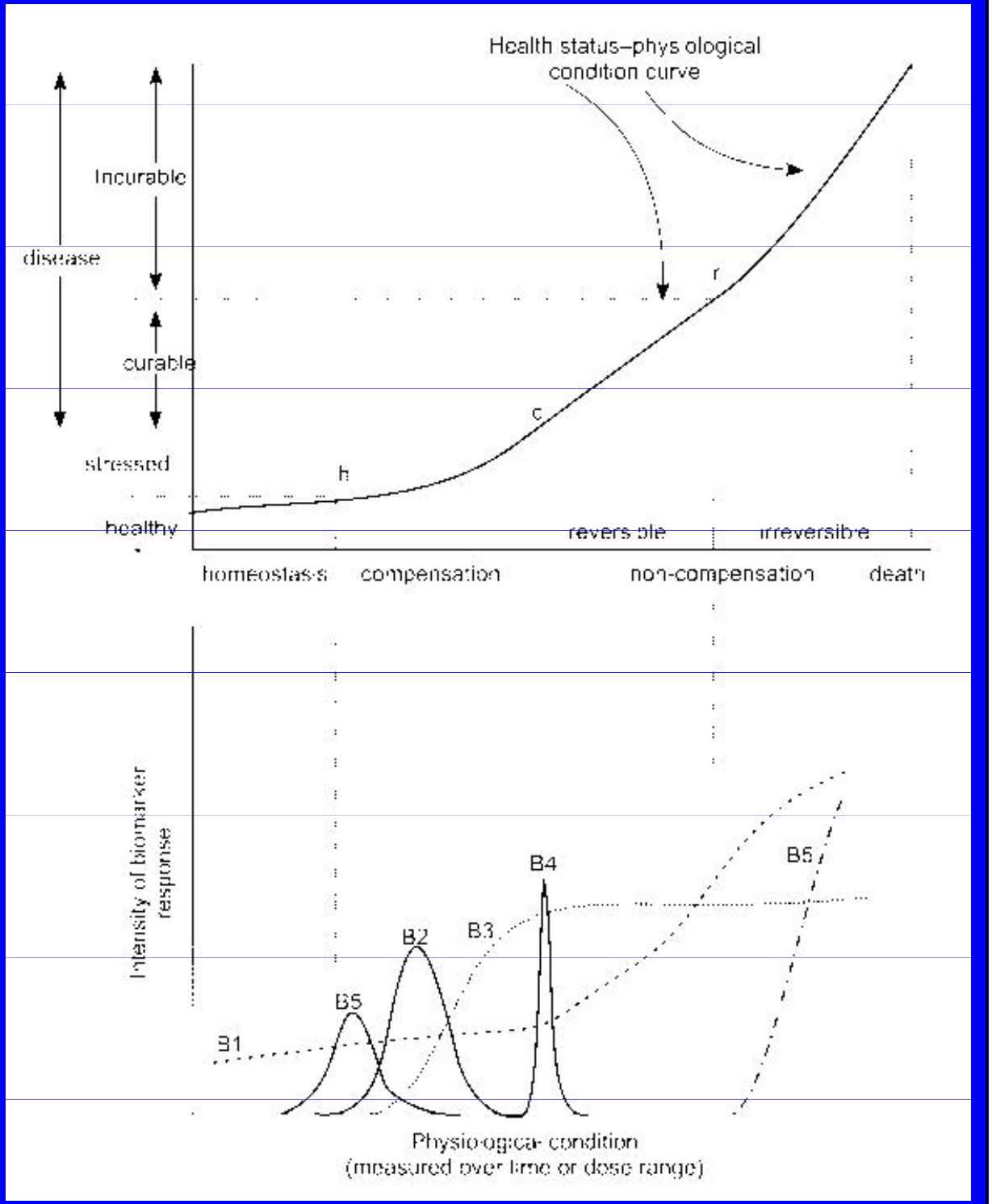
h: homeostatic conditions
c: reversible stage
r: irreversible effects of pollutants

<u>Biomarkers:</u>

- **transitory**
  – B5, B2; short period: B4

- **continuous increase – B3**

- **repeated appearance (B5)**
  – irreversible change



Biomarkers of Exposure

Biomarkers of

- **internal dose** (short / long term)
  - *– Cd in urine, DDE in fat tissues*
  - should be easy to sample (urine, breath)

- **effective dose**
  - the chemical interacted with the target
  - = ADDUCTS

Biomarkers of Exposure - ADDUCTS

Selective aducts (chemical-specific)

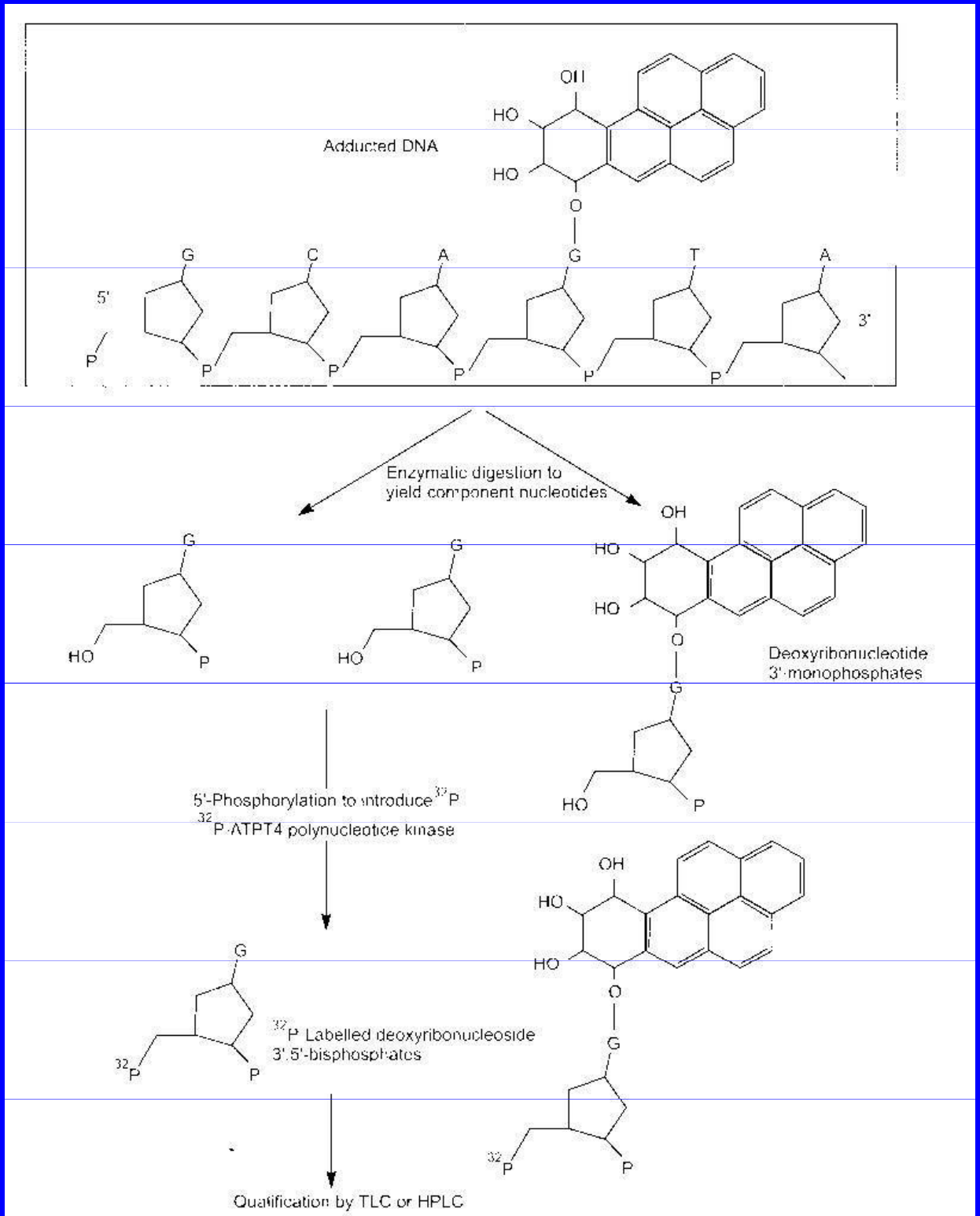
- *DNA aducts: styrene-oxide-O6-guanine*; *N7-guanyl-aflatoxin B1; hemoglobin-pesticides*
- chemical determination (HPLC/GC)

Aselective aducts

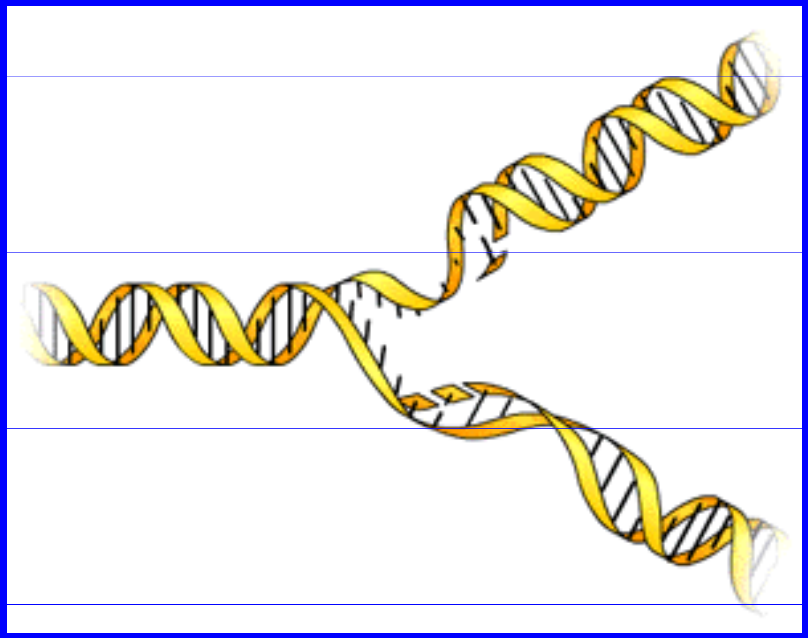
– binding with DNA (proteins) but no info on structure of aduct

- *32P-postlabelling assay*
- *identification of oxy-DNA (8-hydroxy-2´-deoxyguanosine)*
- *DNA-strand breaks – alkaline unwinding assay or comet assay)*

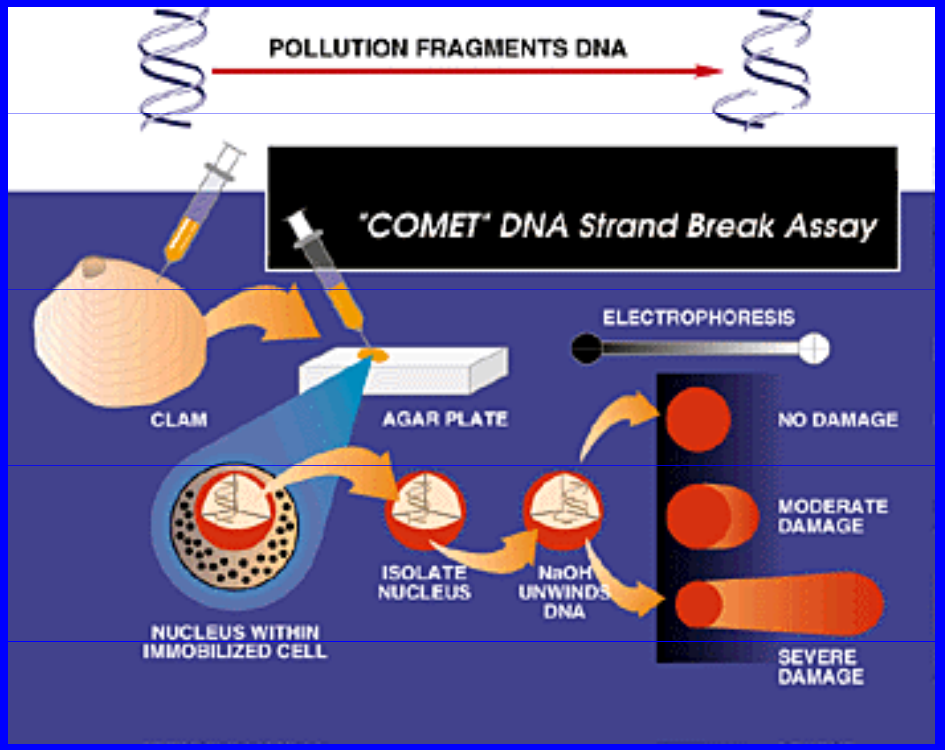
32P-postlabelling assay

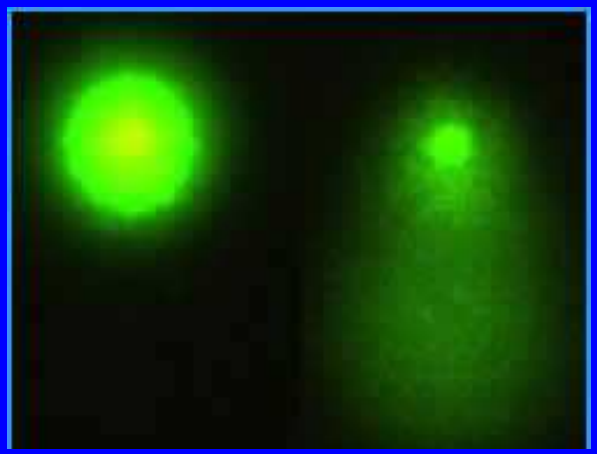


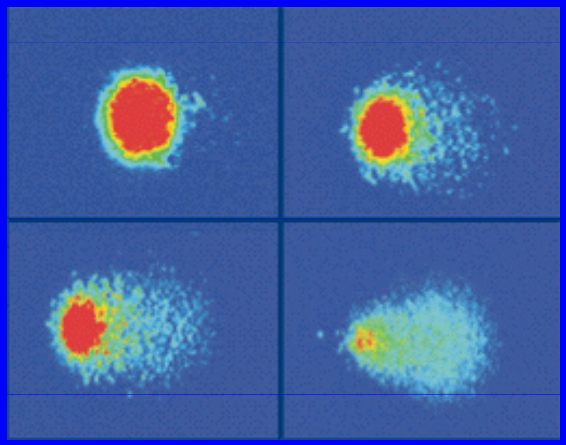
DNA-unwinding assessment



Comet assay







Genetic damage in fish exposed to BaP

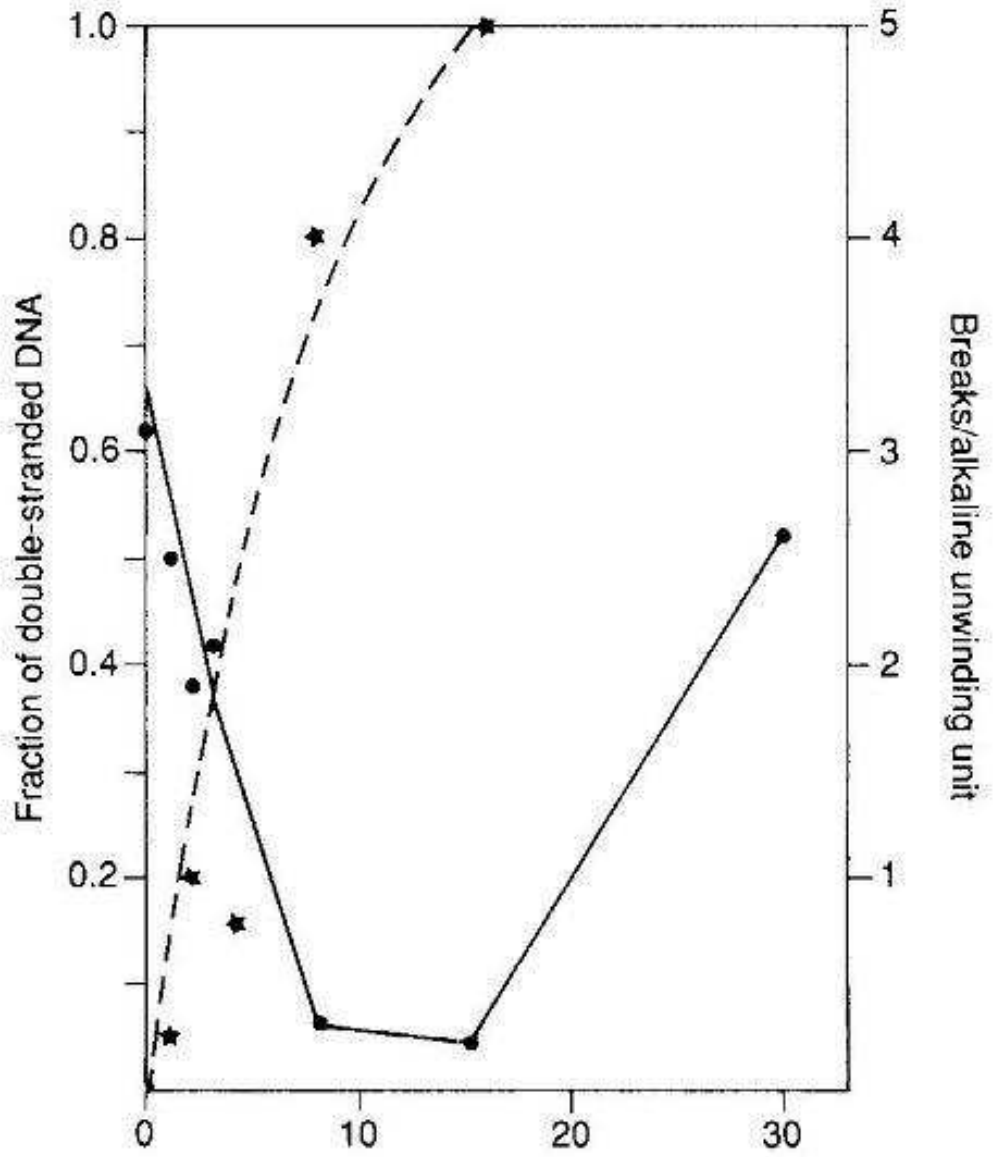
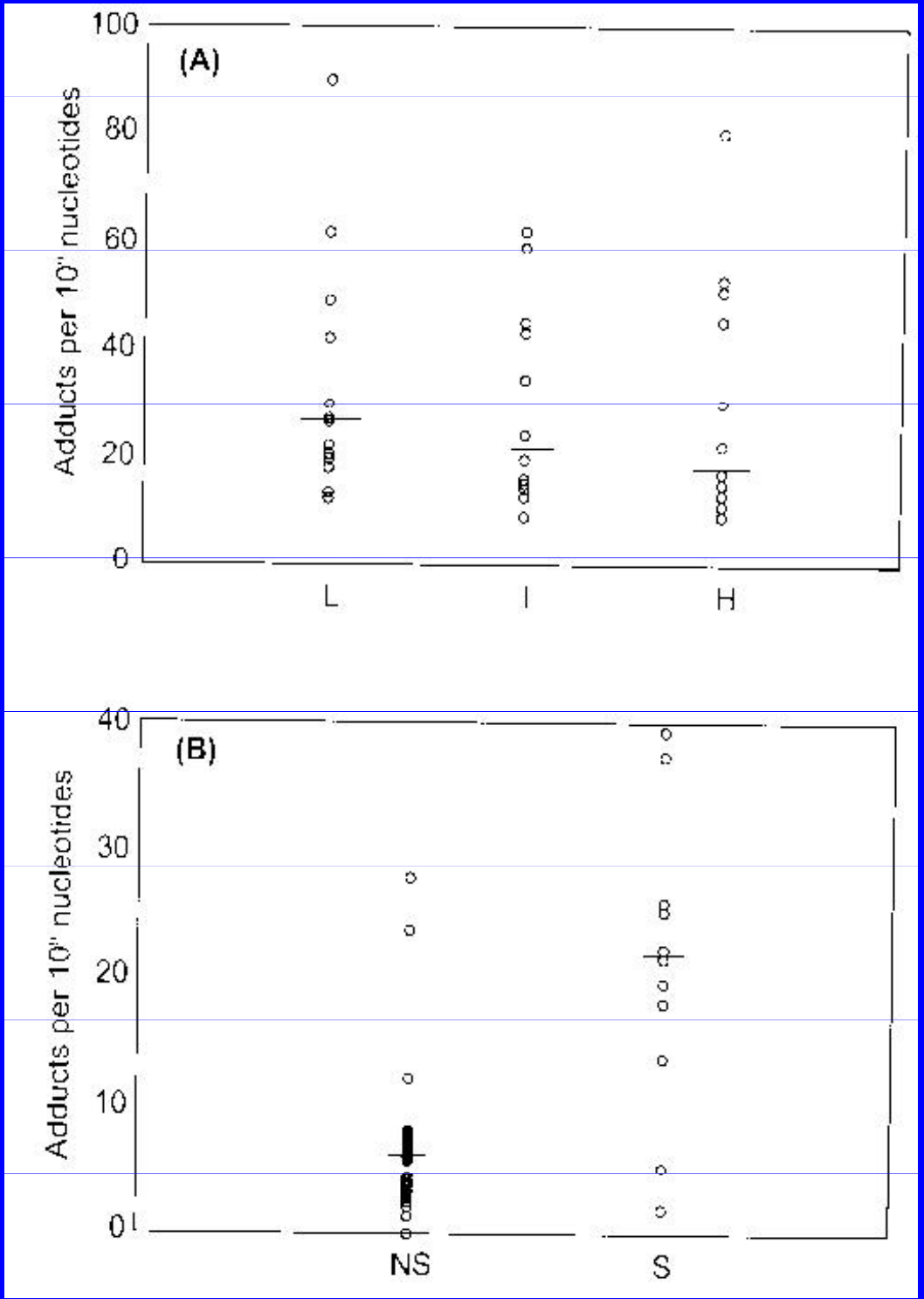


Figure 4.3 Number of breaks and fraction of double-stranded DNA in sunfish exposed to BaP. Shugart (1988).

PAH-DNA adducts



Occup. exposure
(Low / Intermed. / High)

Occupational
Non-exposed (NS)
vs.
Exposed (S)

Table 1 Reported human haemoglobin adduct levels for various xenobiotics

Chemical (type of exposure)	Adduct/analyte	Method	Adduct level (nmol g^{-1} haemoglobin)
N, *N*-Dimethylformamide (occupational)	3-Methyl-5-isopropylhydantoin	Hydrolysis; GC–MS	75–1000 (exposed) 4–12 (control)
Epichlorohydrin (occupational)	*N*-(2, 3-Dihydroxypropyl)valine	Modified Edman; GC–MS	0.020 (exposed smokers) 0.007 (exposed non-smokers) 0.013 (control smokers) 0.007 (control non-smokers)
Acetaminophen (drug overdose)	3-(Cystein-*S*-yl)acetaminophen	Immunoassay	100–4100
PAHs (occupational)	BPDF–Hb	Spectrofluorimetry	0.005–0.139
Ethylene oxide (occupational)	*N*-Hydroxyethylvaline	Modified Edman; GC–MS	5–20 (exposed) 0.1–0.5 (control smokers) 0.01–0.1 (control non-smokers)
Ethene (occupational)	*N*-Hydroxyethylvaline	Modified Edman; GC–MS	0.02
Propylene oxide (occupational)	*N*-Hydroxypropylvaline	Modified Edman; GC–MS	0.05–3.5 (exposed) < 0.02 (unexposed)
Acrylonitrile (smoking)	*N*-Cyanoethylvaline	Modified Edman; GC–MS	0.09
NNK (smoking)	4-Hydroxy-1-(3-pyridyl) butan-1-one	Hydrolysis; GC–MS	0.0015 (smokers) 0.0005 (non-smokers)
4-ABP (smoking)	4-ABP-cysteine	Hydrolysis; GC–MS	0.00025–0.0025 (smokers) 0.00005–0.0005 (non-smokers)
Acrylamide (occupational, smoking)	*N*-(2-Carbamoylethyl)valine	Modified Edman; GC–MS	9.5 (production workers) 0.054 (laboratory workers) 0.116 (smokers) 0.031 (non-smokers)
Butadiene (occupational)	*N*-(2,3,4-Trihydroxybutyl)valine	Modified Edman; GC–MS	0.010–0.014 (exposed) 0.002–0.003 (control)
Styrene (occupational)	2-Phenylethanol	Cleavage with Raney nickel, GC–MS	3.7–8.0 (exposed) 2.0–8.6 (control)

Biomarkers of susceptibility

Metabolism

- variability in specific enzymes
- susceptibility to modify toxicants: *N-acetylation of arylamines – NAT2*
- null genotypes for conjugation enzymes (*GSTM1*)

Genotype

- familial cancers & susceptibility to genotoxins

Biomarkers of susceptibility

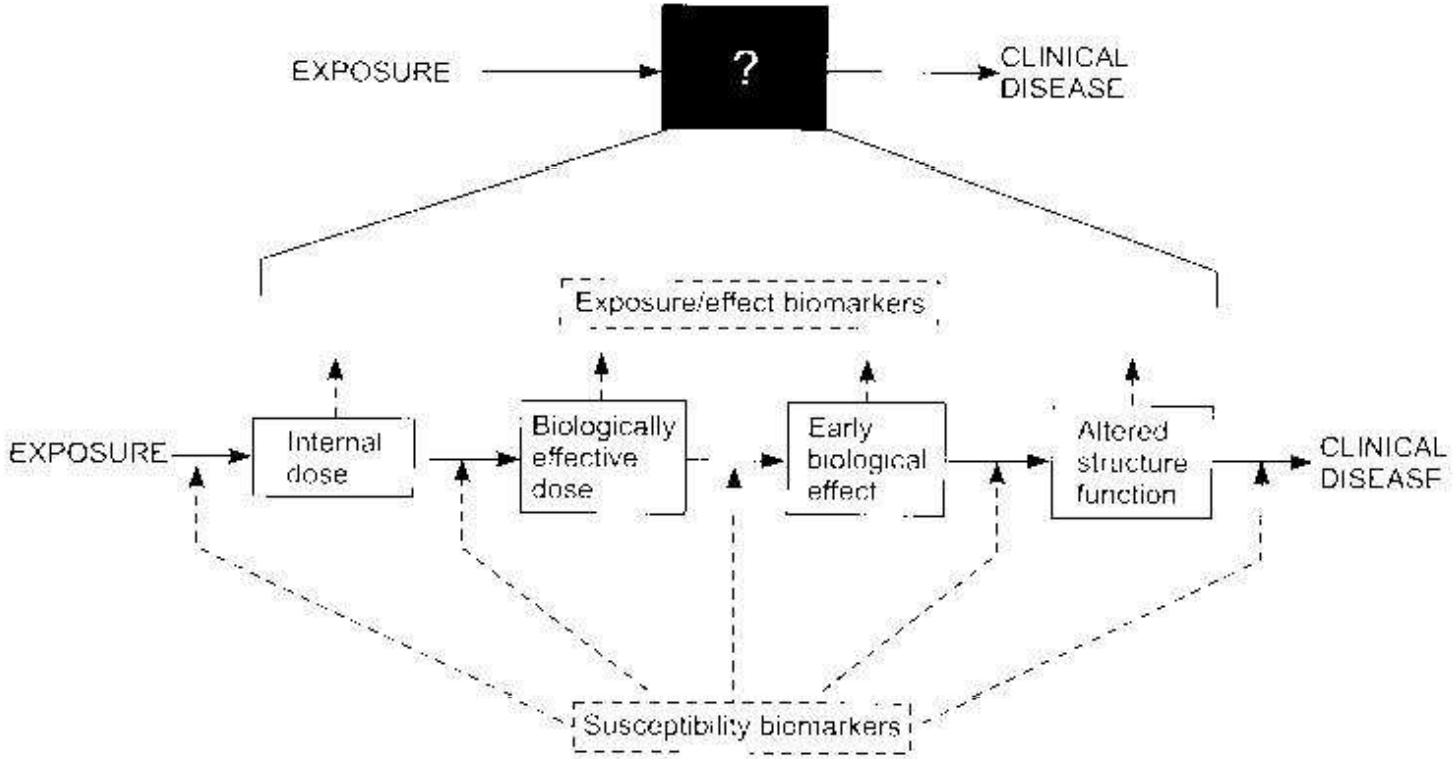


Figure 1 The biomarker paradigm linking exposure with disease and showing expansion of the classical epidemiological 'black box' to reveal discrete mechanistic stages. Reprinted with permission from *Environ. Sci. Technol.* (1997) **31**, pp. 1837–1848. Copyright 1997 American Chemical Society.

Biomarkers of susceptibility

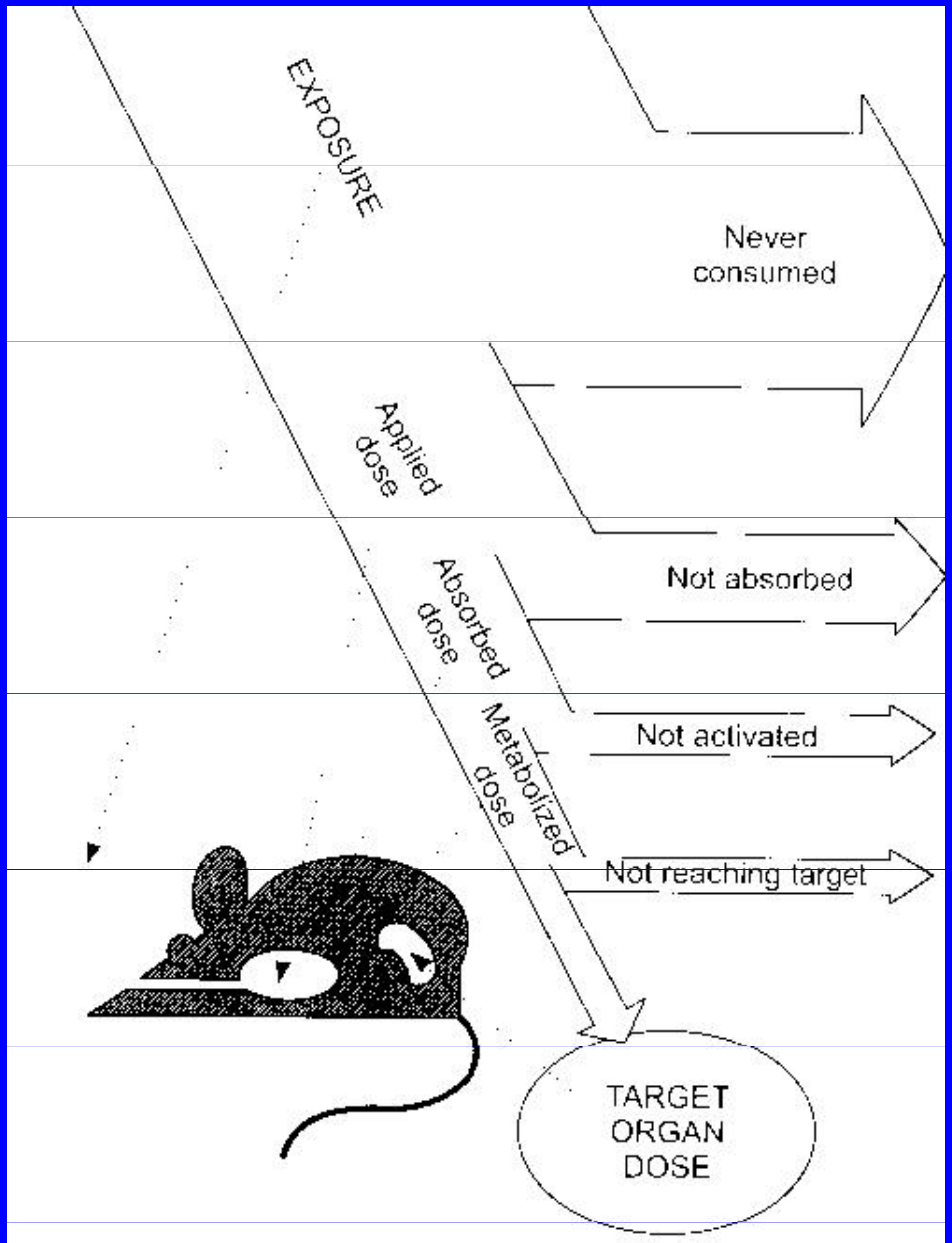


Figure 2 Representation of the relationships between ambient exposure and critical target dose and the progressive decrease in effective exposure due to various biological barriers. Source: *Low-Dose Extrapolation of Cancer Risks: Issues and Perspectives*, p. 188. Used with permission. © 1995 International Life Sciences Institute, Washington, DC, U.S.A.

In vivo biomarkers of effects / response

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

Behaviour and Clinical biomarkers
Pathology
Clinical chemistry
Enzymatic changes
Protein synthesis
Oxidative stress markers

+ Human: Excretory products in urine

Tumor genes and tumor markers

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

suppressor genes *p53, Rb*

Behaviour and clinical biomarkers

Parameters evaluated

- body weight
- food consumption
- fitness & welness

Interpretation

: ? biomarkers ? effects already demonstrated *in vivo*

- biomarkers of existing serious stress / intoxication

Behaviour 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).

Pathology

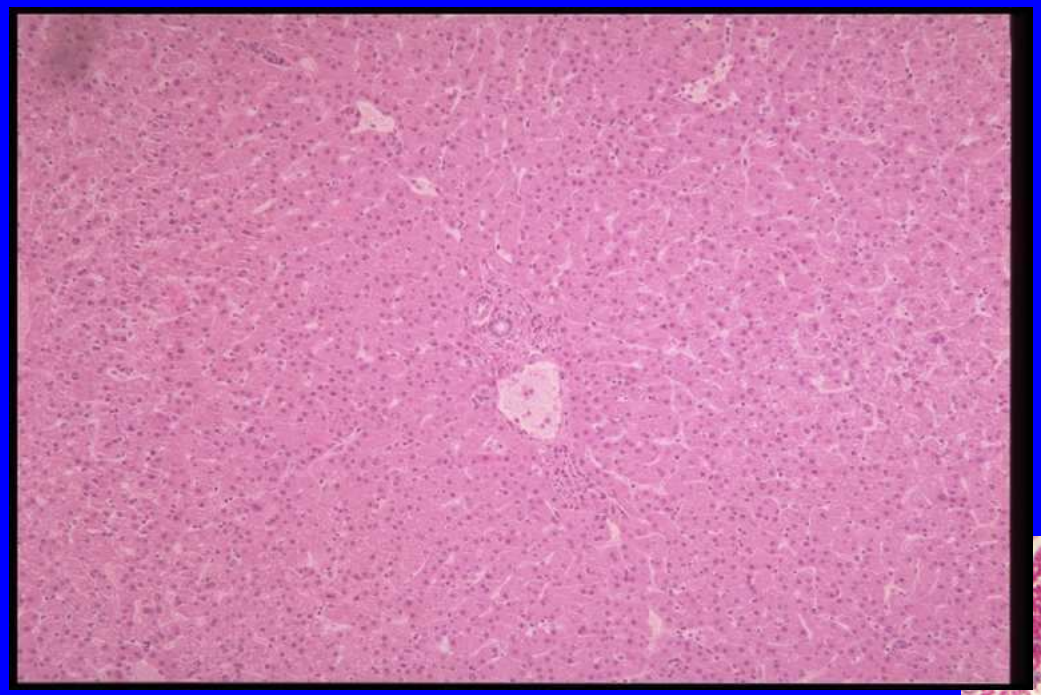
(-) Destructive methods, Time consuming, Professional requirements
(+) High relevance – organ/tissue changes

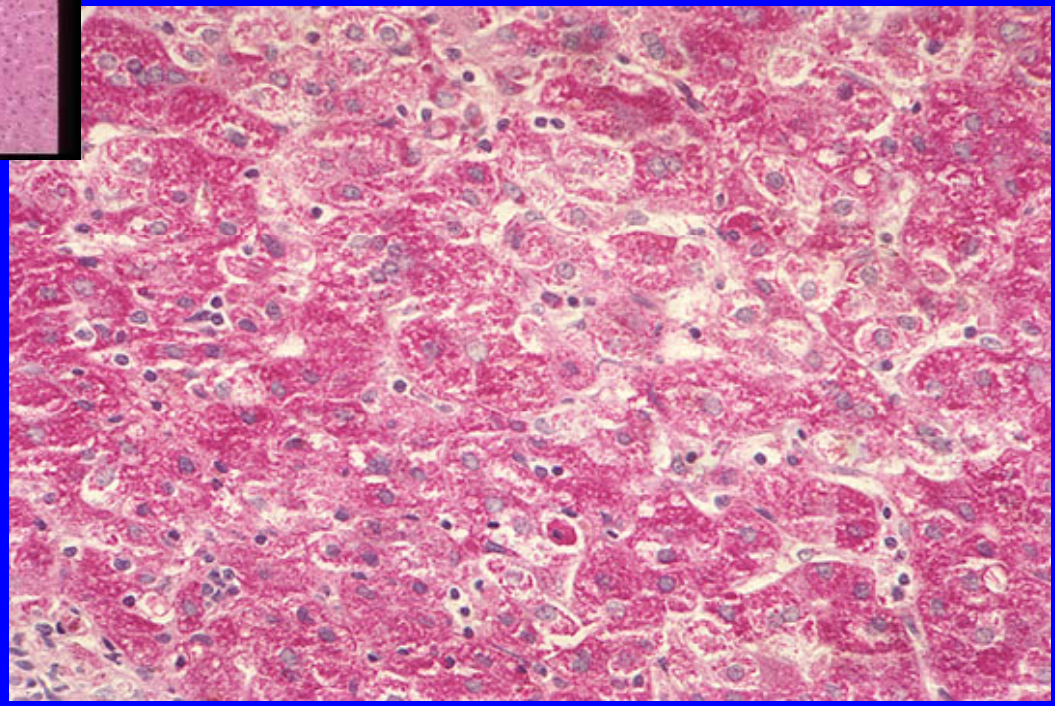
- **<u>microscopy of internal organs</u>**
  : non-specific changes in internal organs
  : specific changes in liver (dioxin-like POPs, cyanobacterial toxins)
  : intersex / imposex formation (xenoestrogenicity)

- **<u>immunohistochemistry & microscopy</u>**
  : determination of specific changes
  : Fluorescein (FITC)- labeled antibodies (Ab) applications
    - determination of vitellogenin in male organs (anti-Vtg Ab)
    - autoimmunity (anti-nuclear Ab, ANA, in exposed organisms)

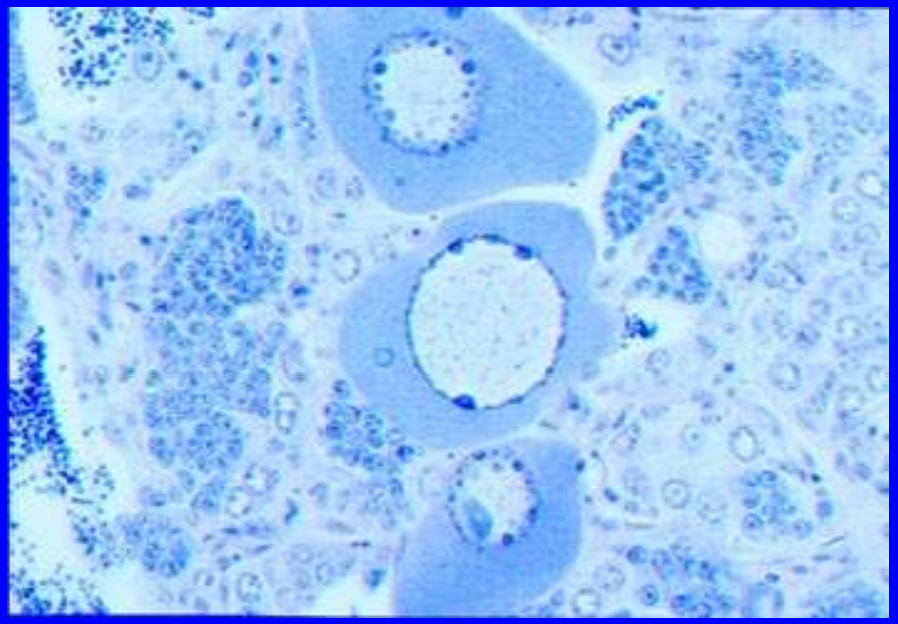
- **<u>chromosomal abnormalities & micronuclei evaluation</u>**
  : karyotype biomarkers
  : non-destructive (blood samples; plant tissues)

Pathology - Liver damage by microcystins

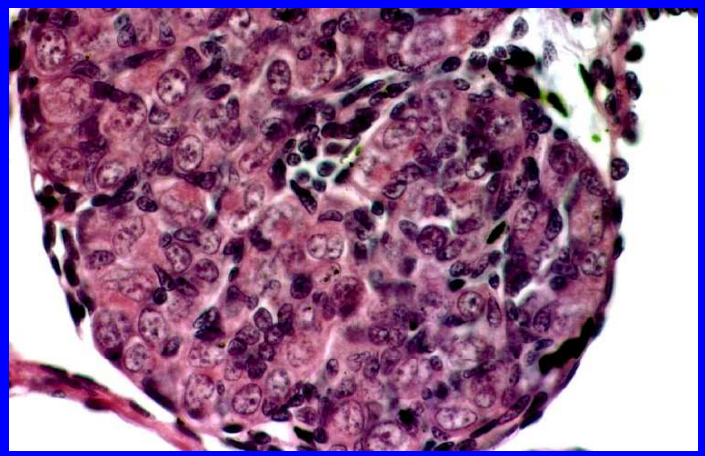


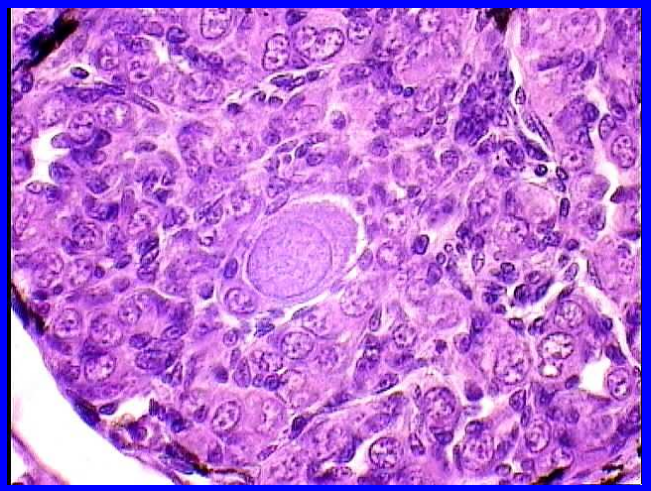


Pathology – Intersex microscopy



Oocytes
in testicular tissue





Immunohistochemical determination of Vtg in male fish

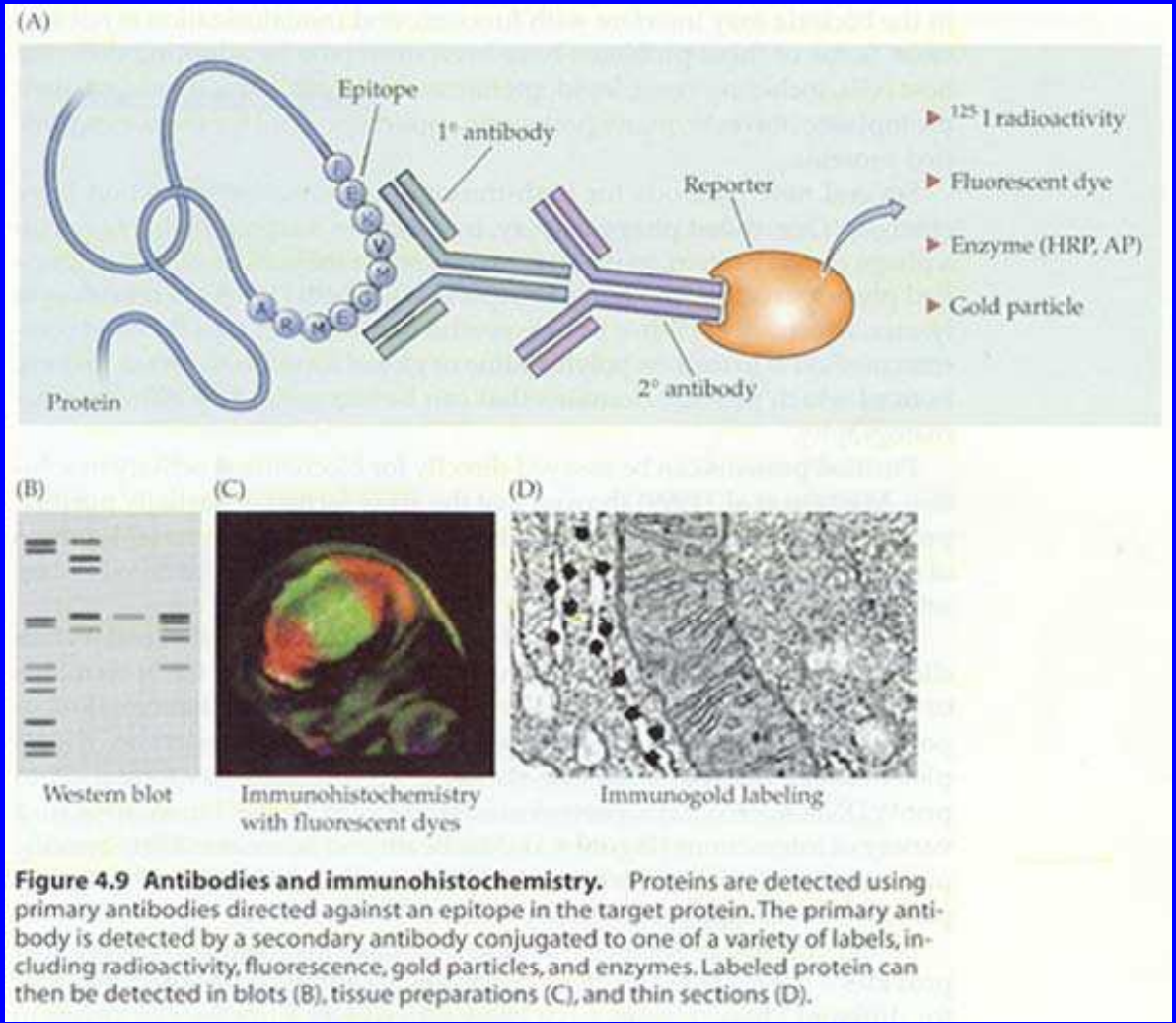
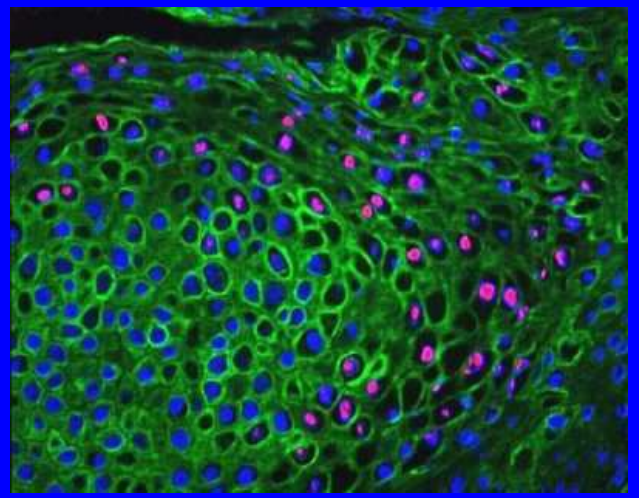
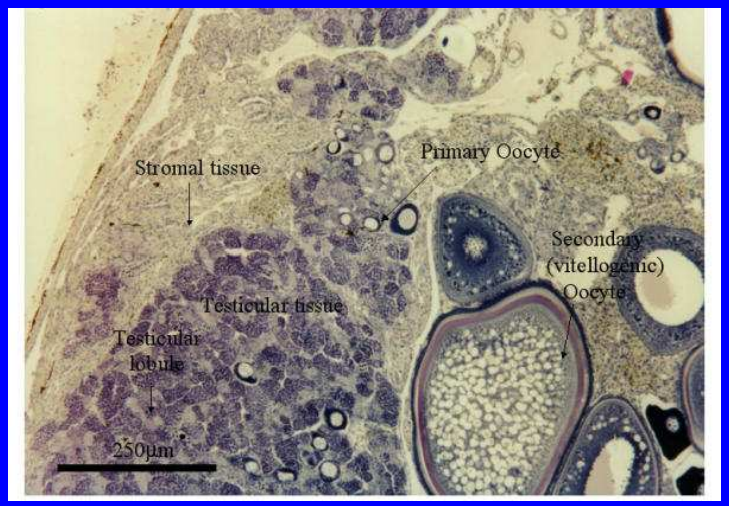
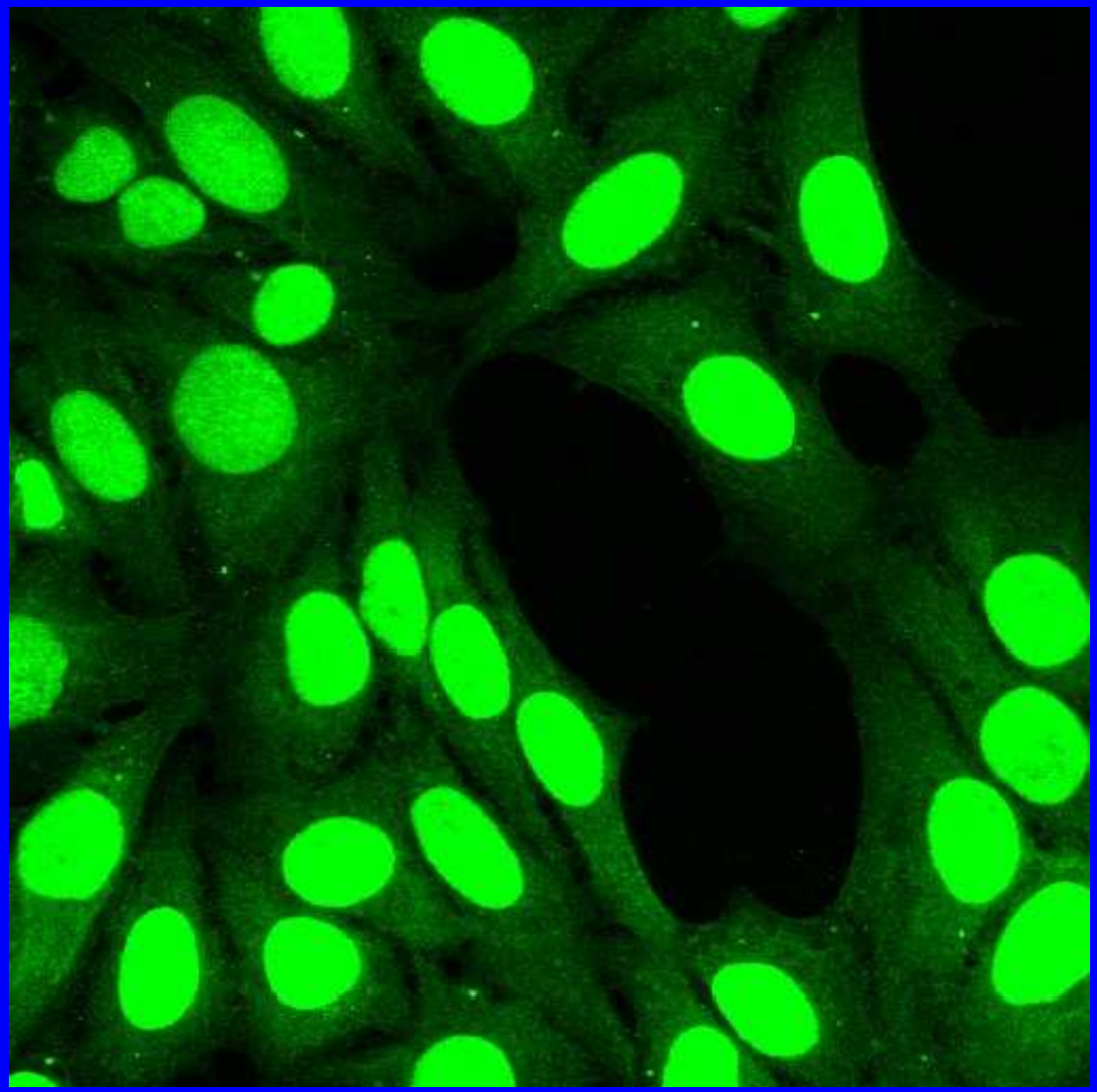


Figure 4.9 Antibodies and immunohistochemistry. Proteins are detected using primary antibodies directed against an epitope in the target protein. The primary antibody is detected by a secondary antibody conjugated to one of a variety of labels, including radioactivity, fluorescence, gold particles, and enzymes. Labeled protein can then be detected in blots (B), tissue preparations (C), and thin sections (D).

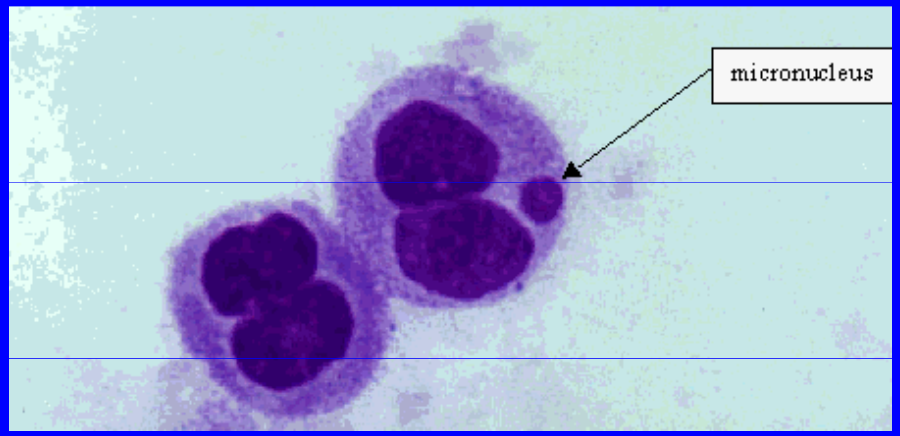


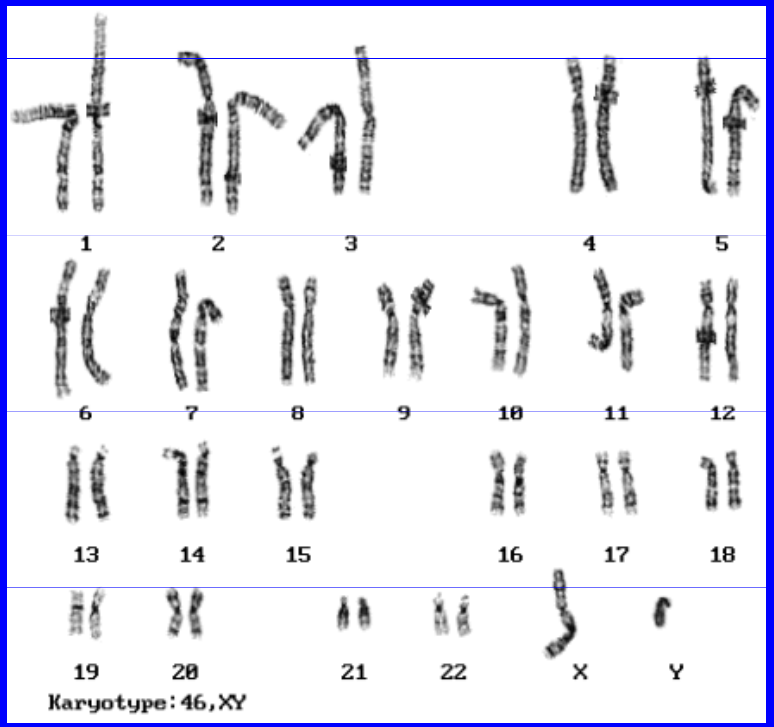


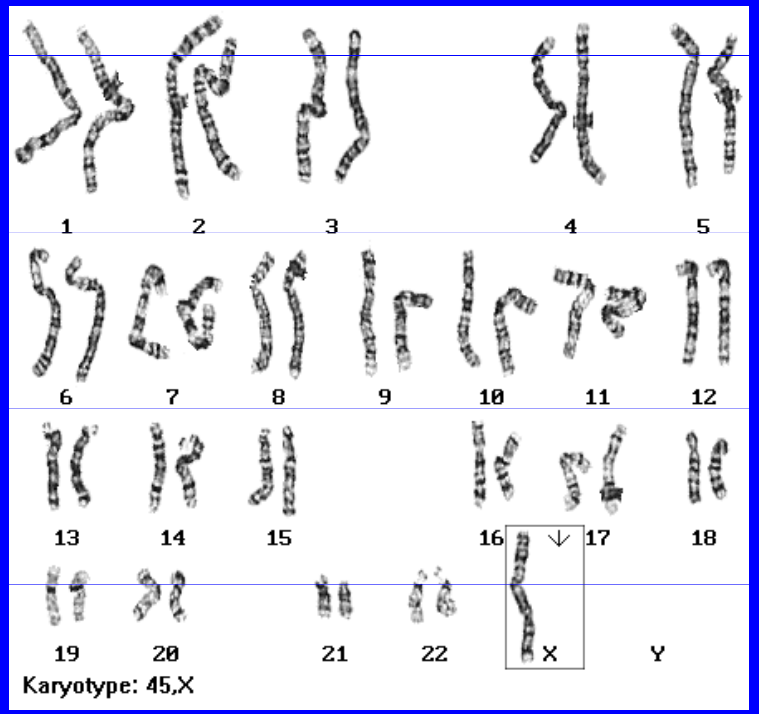
Immunohistochemistry of ANA in autoimmune serum



Chromosomal aberations
Micronuclei determinations







Clinical chemistry

Non-destructive

Often specific interpretation

- determination of enzymatic activities in blood

- response to tissue/organ damage

- muscle damage: **creatine kinase** in serum
  : isozymes - tissue specific (brain, muscle, heart);
- heart attack – isozymes of **lactate dehydrogenase** (LDH)
- liver damage – AST (....), ALT (....) in blood
  : cyanotoxins, dioxin-like POPs

Example – changes in rat serum enzymes after CCL4 exposure

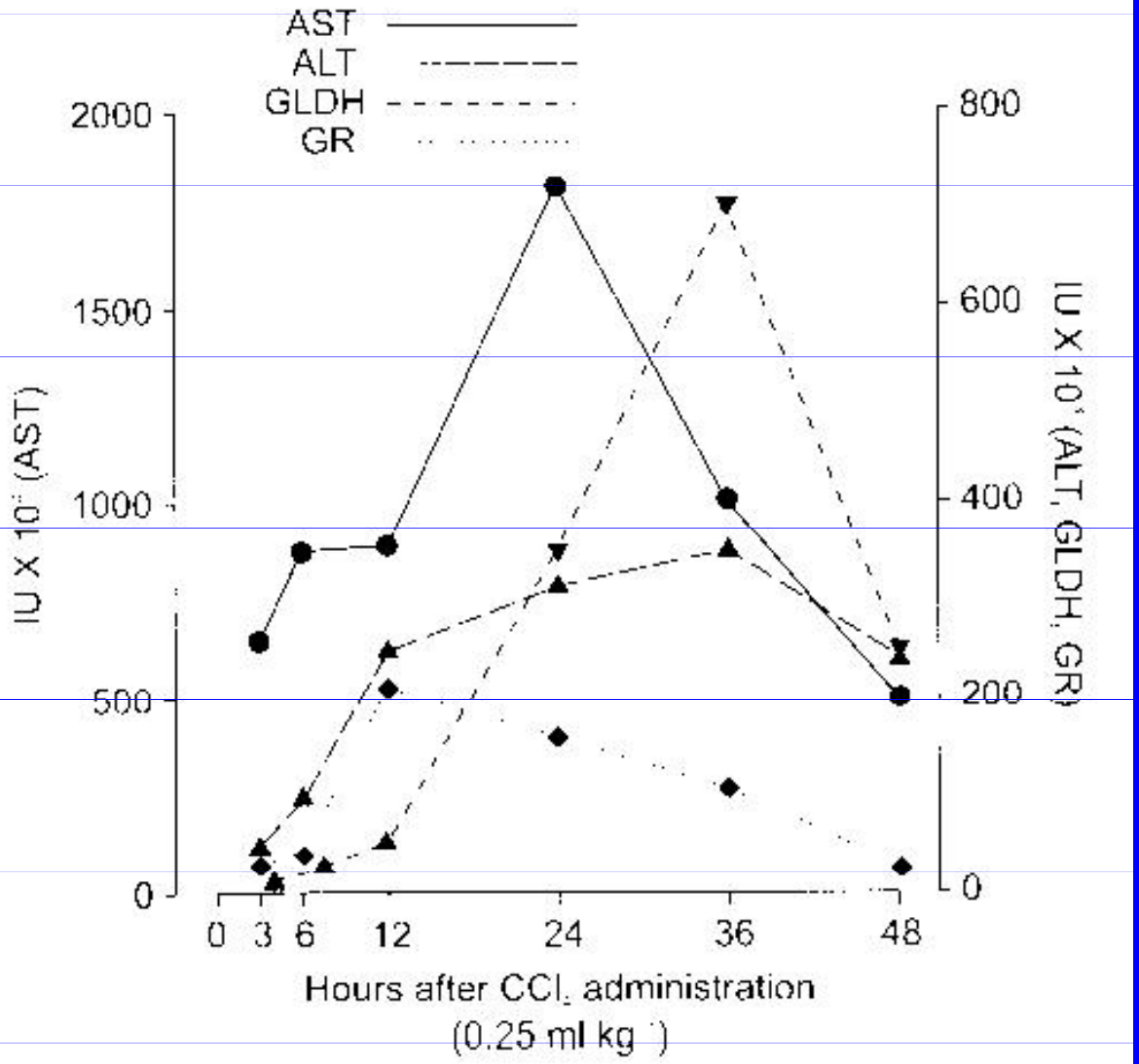


Figure 3 Serum enzyme levels in rats following dosing with carbon tetrachloride (CCl_4, 0.25 ml kg^{-1}). Redrawn from Zimmerman (1978).

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)

Enzymatic 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)

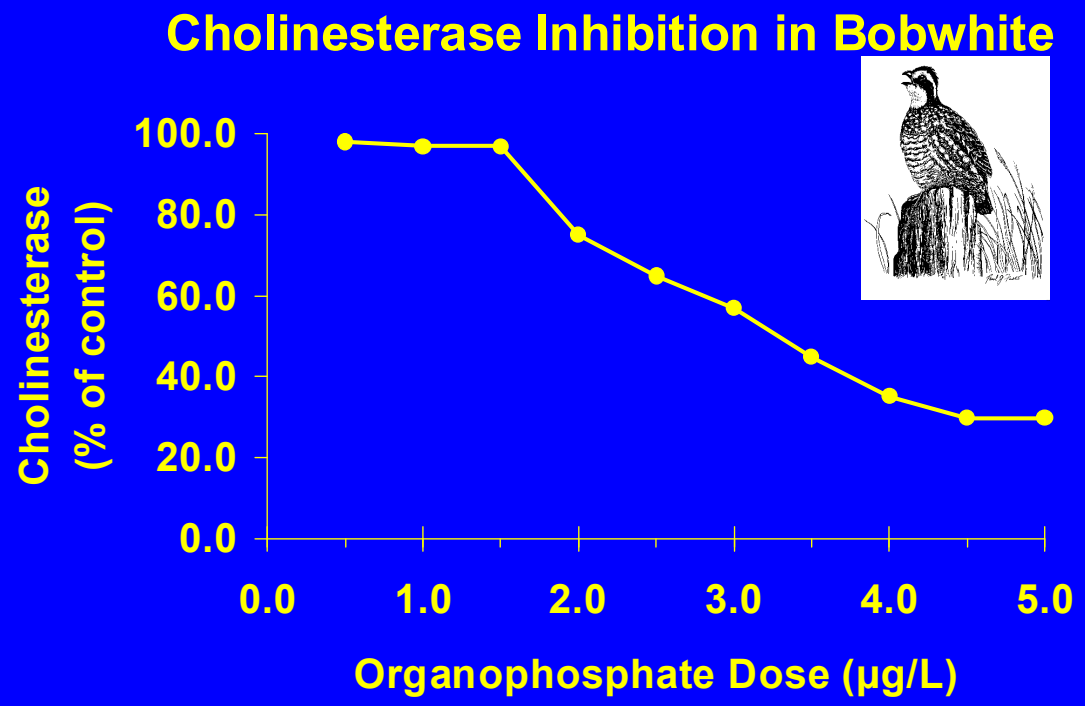
(+) Rapid enzymatic assays, specific responses

(-) Some ~ EXPOSURE biomarkers

AcChE inhibition 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

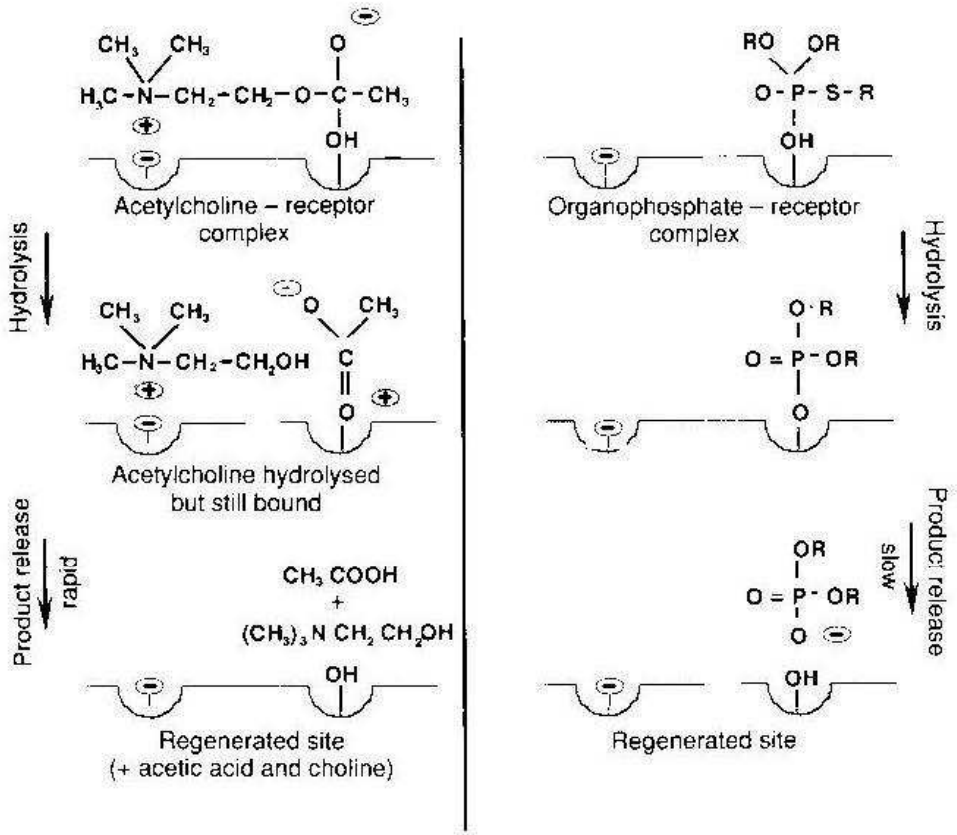


Figure 2.2 Mode of action of inhibition of acetylcholinesterase.

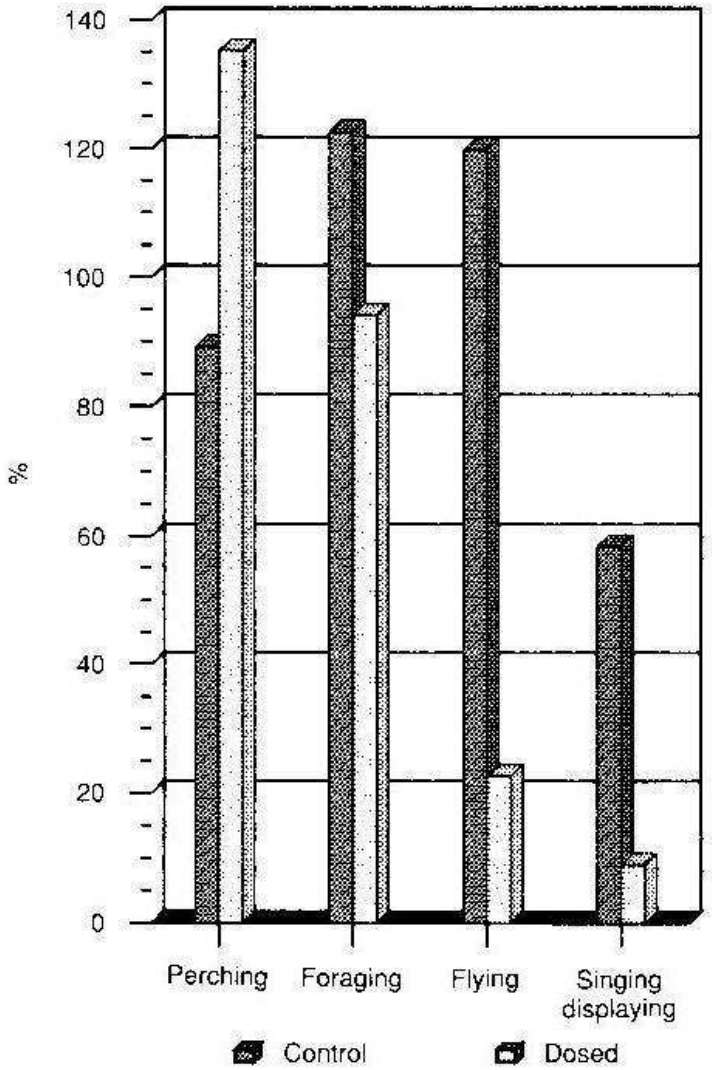


Figure 2.4 Effect of OP on behaviour of starlings. After Grue and Shipley (1981).

AcChE inhibition
mechanism & effects in birds

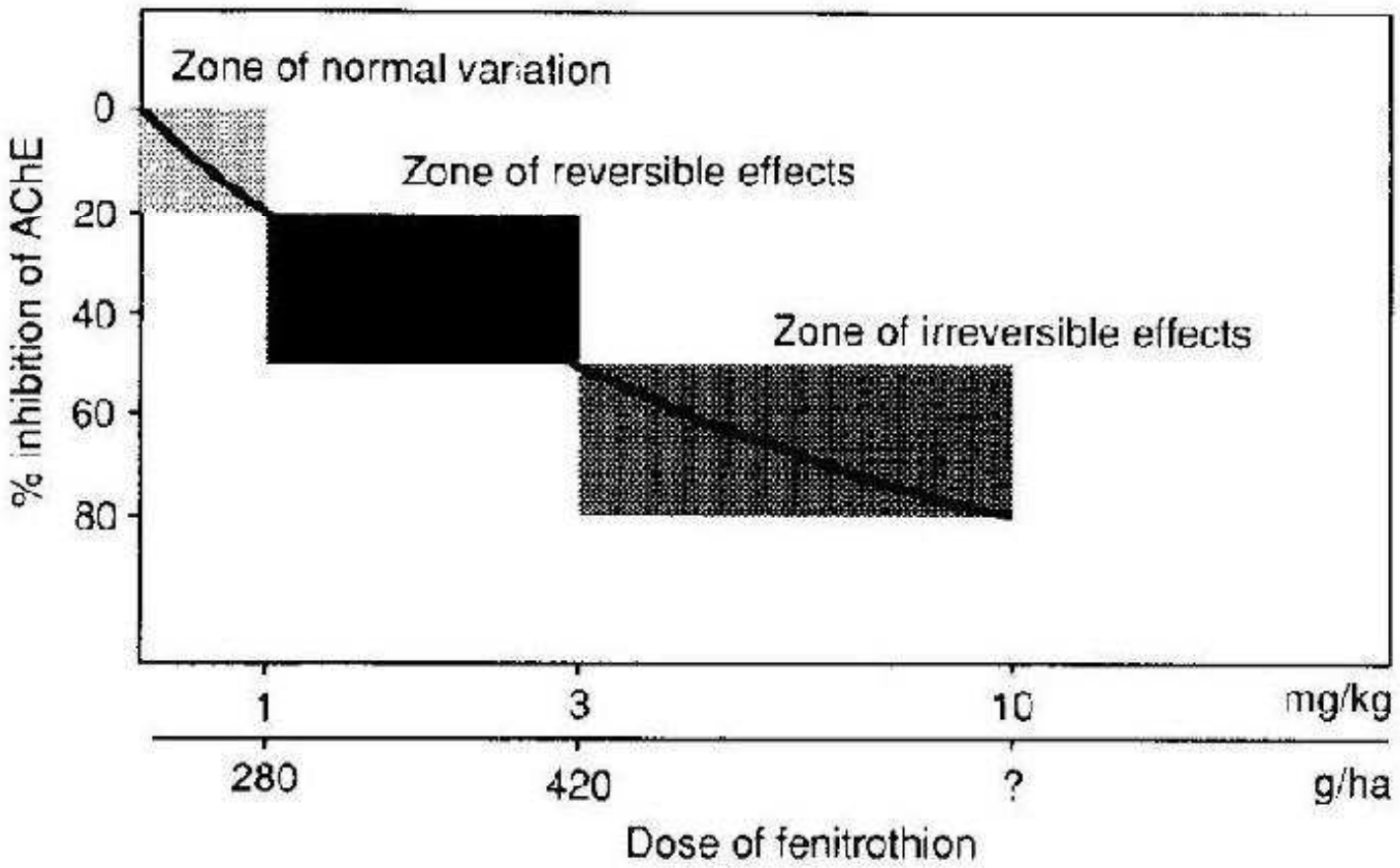


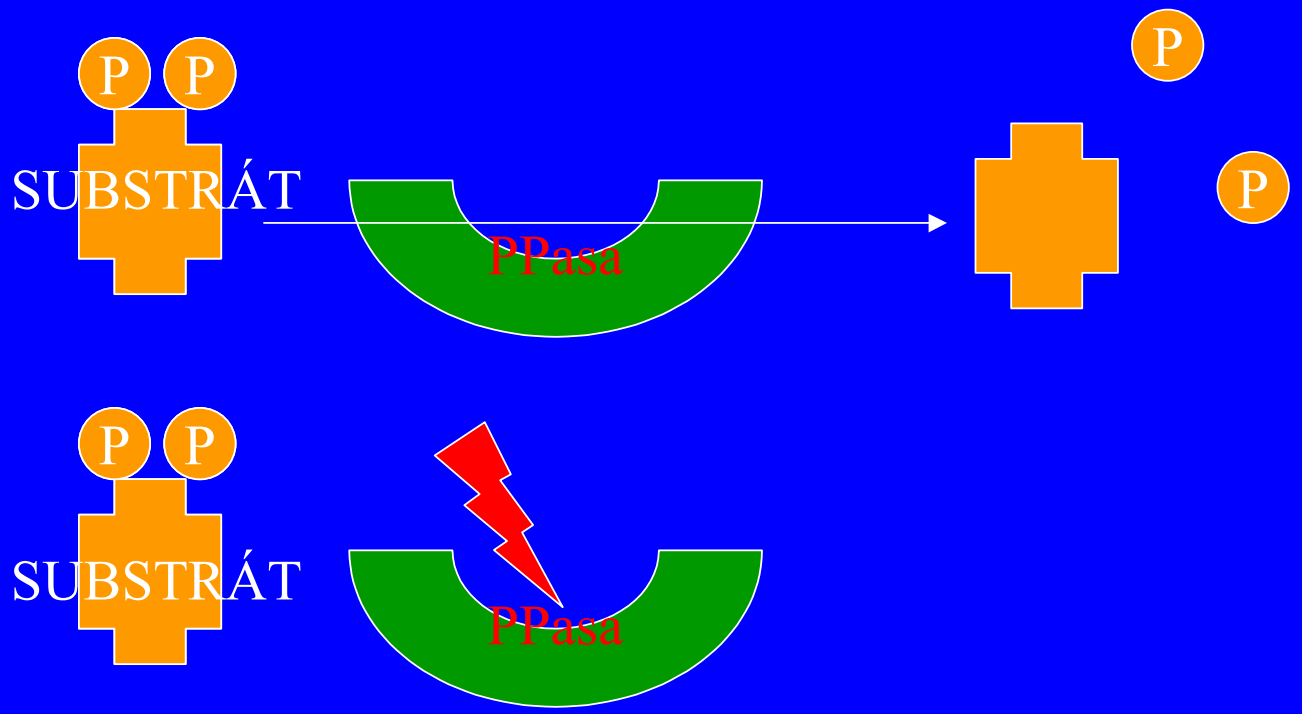
Figure 10.2 Dose response of AChE inhibition.

PPase 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

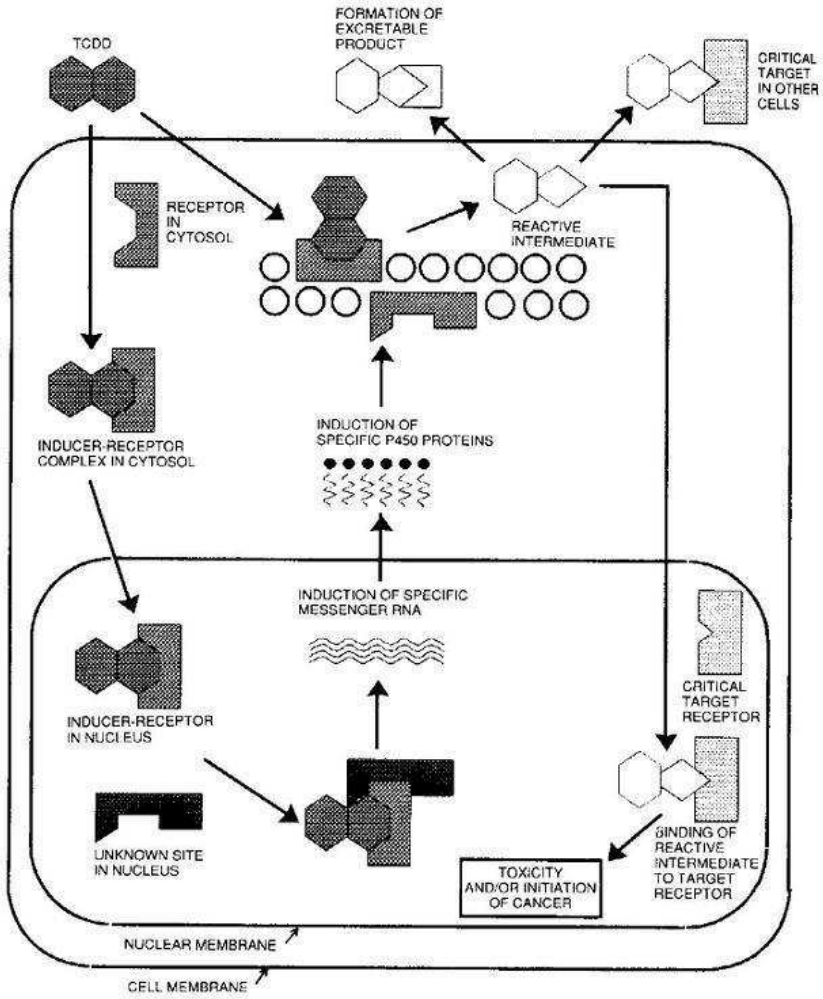


Figure 5.1 Diagram of MFO system. Nebert and Gonzalez (1987).

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

- endoplasmic reticulum (membrane bound) CYPs – mirosomal vesicles (S9-fraction)

substrate: Ethoxyresorufin
-> Oxidation by CYP1A1 -> Fluorescence
EthoxyResorufin-O-Deethylase activity EROD

- other substrates: CYP isozymes: BROD, MROD, PROD ...

AHH (ArylHydrocarbon Hydroxylase) ~ similar method for MFO
- substrate: Benz[a]pyrene -> oxidation

Biomarker of organic pollution (exposure & effects)

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

: often used in environmental studies

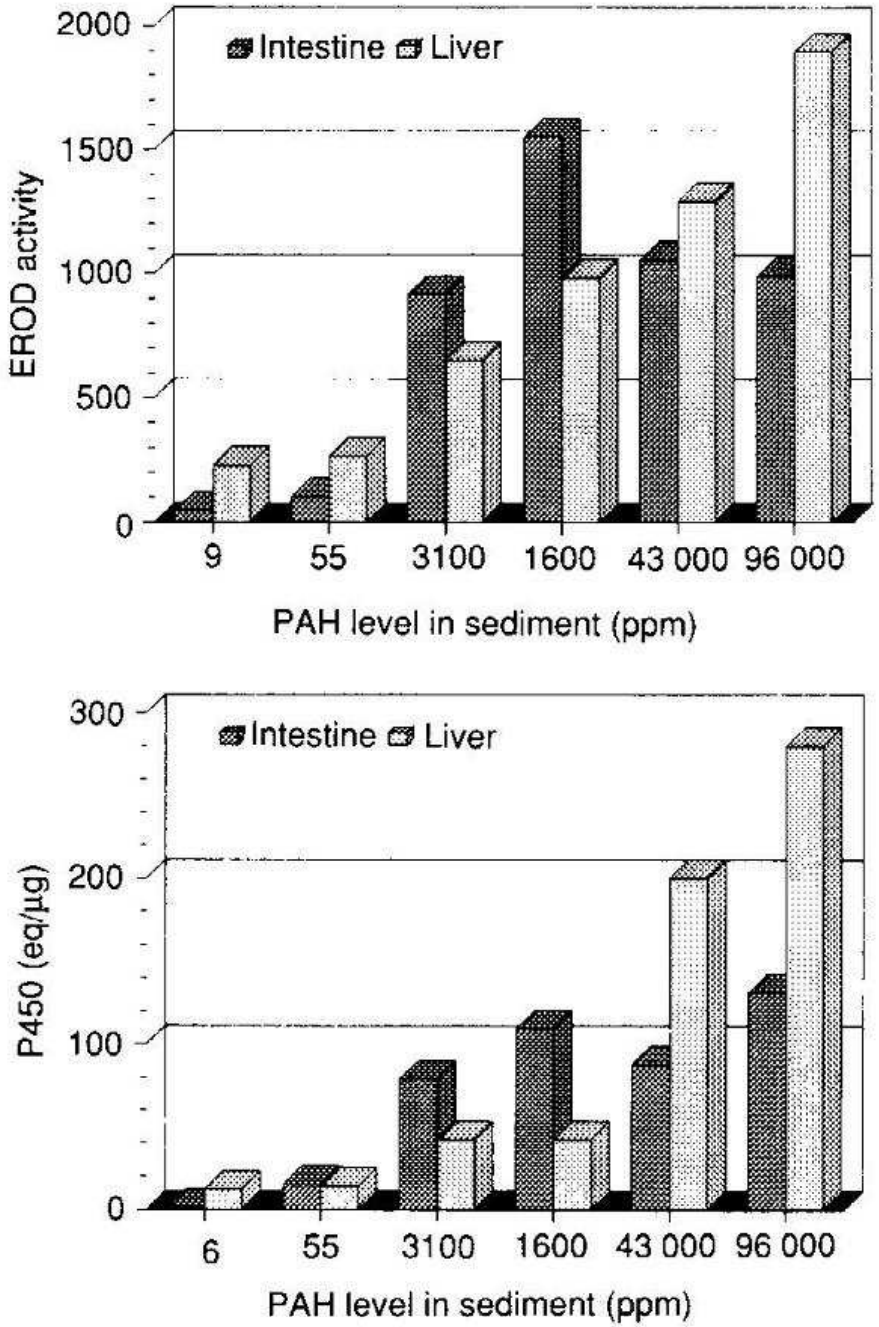


Figure 5.6 Relationship of sediment concentration of PAHs to EROD activity in liver and intestine of spot. After Van Veld *et al.* (1990).

Locality:
Reference Exposed

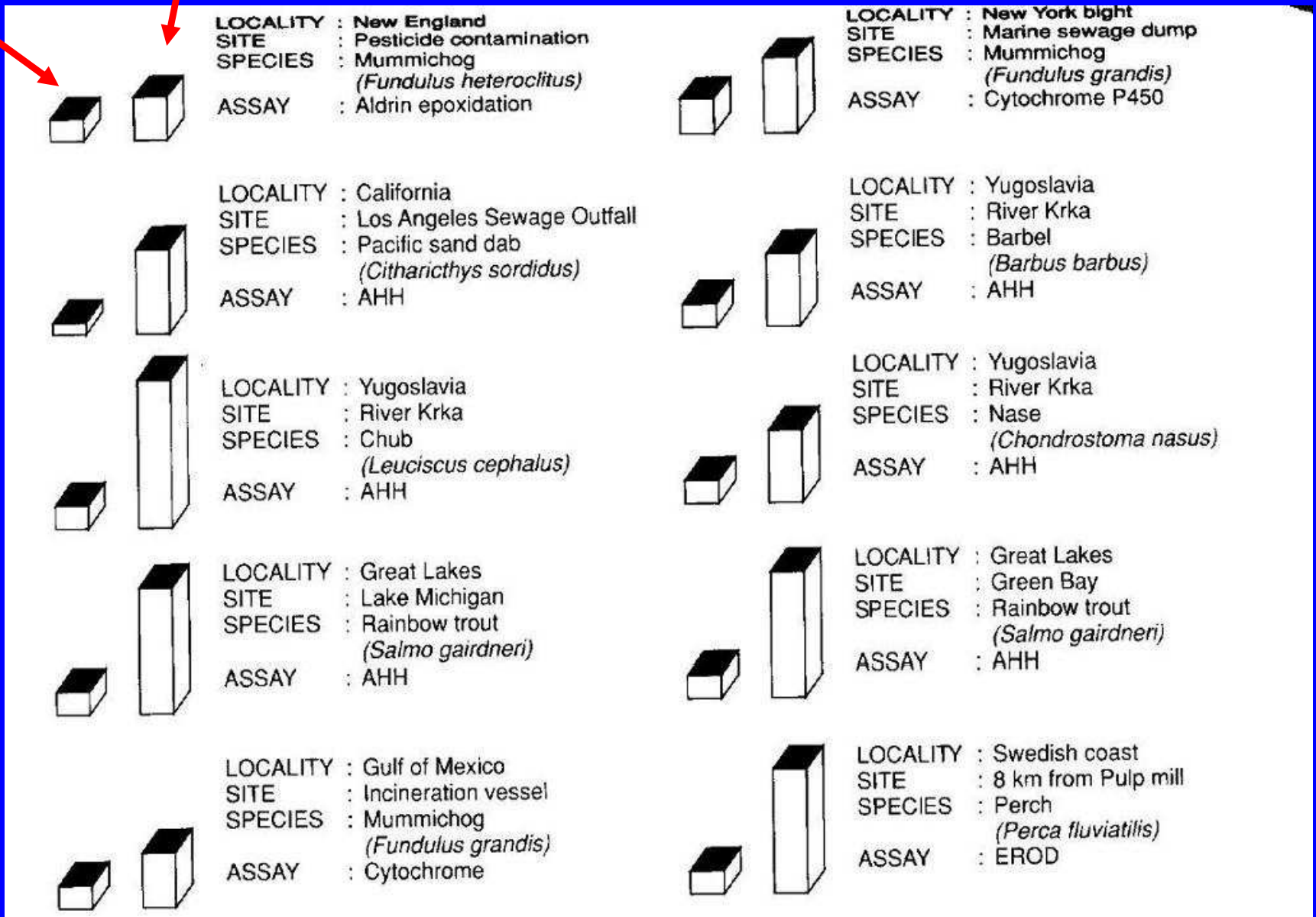
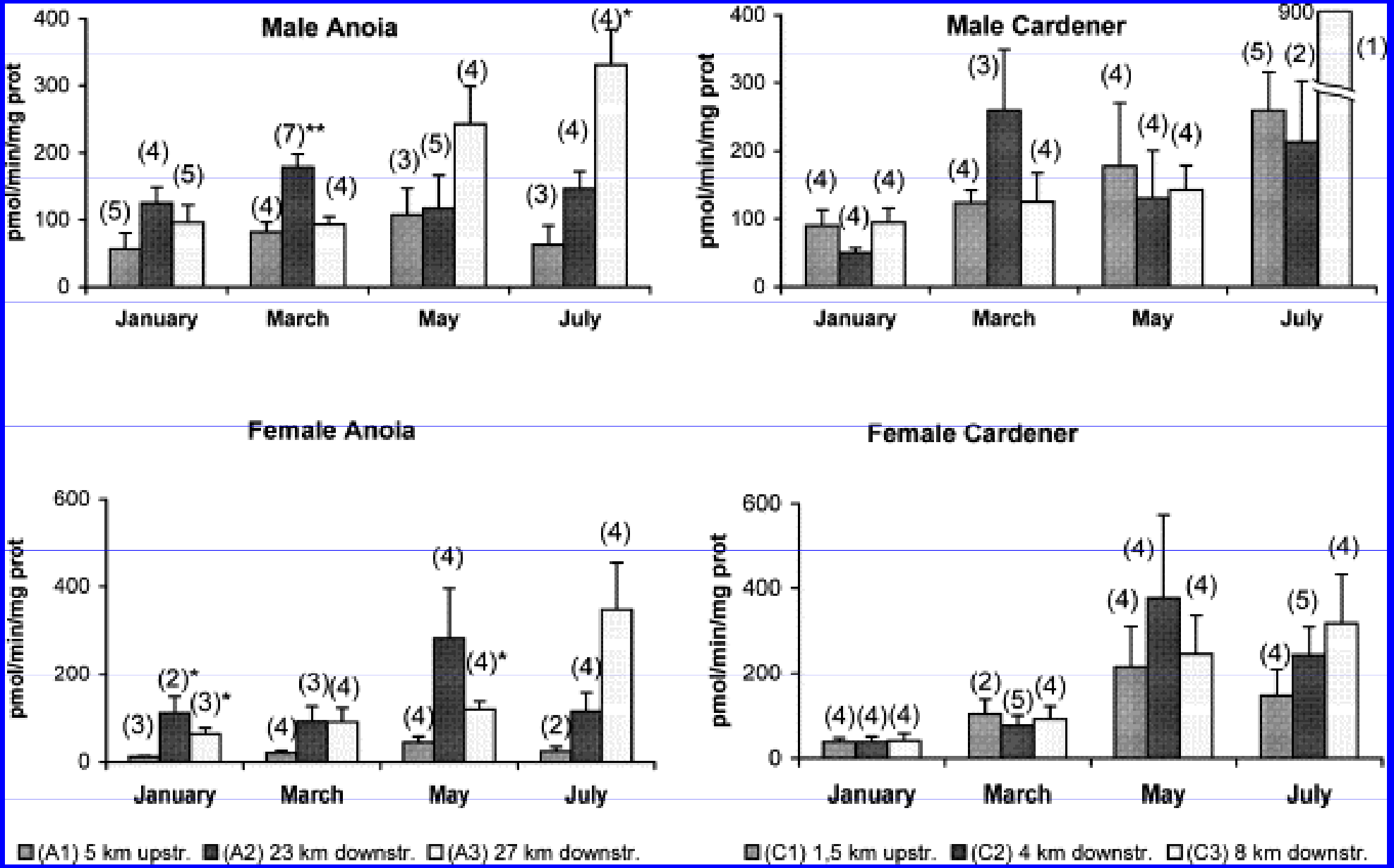


Figure 5.5 MFO changes in fish exposed to organic contamination. The proportion of either enzyme or cytochrome P450 levels detected at reference (short towers) and experimental sites (long towers) is presented in schematic form. All differences between reference and experimental sites were statistically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

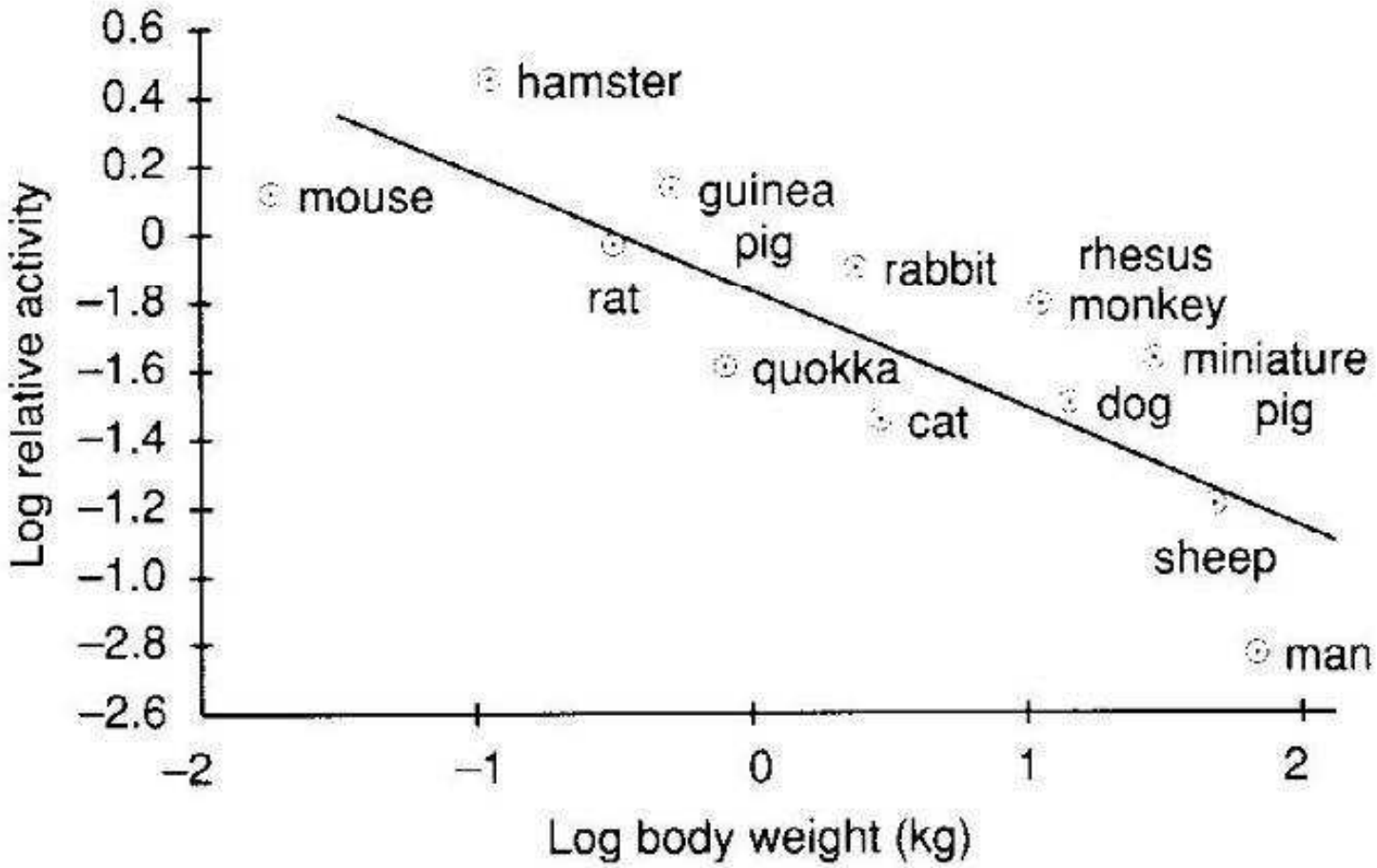


Figure 5.3 Relationship of body weight to MFO activity in mammals. Walker (1978 and 1980).

Potencies to induce CYPs (AhR)

PCDD/Fs and co-planar PCBs

- induction of MFO is structure-dependent; potencies & toxicities among compounds differ
- international agreement on **TEF/TEQ approach** to characterize dioxin-toxicity in environmental samples (WHO)
- each compound (only few selected in WHO agreement) relative potency (TEF) related to 2,3,7,8-TCDD

2,3,7,8-TCDD	TEF = 1
Several other PCDD/Fs	0.1-1
PCBs	10^{-5} – 0.1 (No. 77, 126)

- species-specific TEFs for humans / fish / birds

- chemical analyses of samples
  => SUMA (concentrations x TEF) = TEQ (ng TCDD / sample)
- EASY comparison of sample contamination

TEFs for selected PCDDs

CONGENER	TOXIC EQUIVALENCY FACTOR (TEF)		
	HUMANS/ MAMMALS	FISH [a]	BIRDS [a]
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	1	1	1 [f]
1,2,3,4,7,8-HxCDD	0.1 [a]	0.5	0.05 [f]
1,2,3,6,7,8-HxCDD	0.1 [a]	0.01	0.01 [f]
1,2,3,7,8,9-HxCDD	0.1 [a]	0.01 [e]	0.1 [f]
1,2,3,4,6,7,8-HpCDD	0.01	0.001	<0.001 [f]
OCDD	0.0001 [a]	-	-

TEFs for PCBs

Congener Number	IUPAC Chlorobiphenyl Prefix	1994 WHO TEFs(1)	1997 WHO TEFs(2)		
			Humans/ Mammals	Fish	Birds
PCB-77	3,3',4,4'-Tetra-	0.0005	0.0001	0.0001	0.05
PCB-81	3,4,4',5-Tetra-	--	0.0001	0.0005	0.1
PCB-105	2,3,3',4,4'-Penta-	0.0001	0.0001	<0.000005	0.0001
PCB-114	2,3,4,4',5-Penta-	0.0005	0.0005	<0.000005	0.0001
PCB-118	2,3',4,4',5-Penta-	0.0001	0.0001	<0.000005	0.00001
PCB-123	2,3',4,4',5'-Penta-	0.0001	0.0001	<0.000005	0.00001
PCB-126	3,3',4,4',5-Penta-	0.1	0.1	0.005	0.1
PCB-156	2,3,3',4,4',5-Hexa-	0.0005	0.0005	<0.000005	0.0001
PCB-157	2,3,3',4,4',5'-Hexa-	0.0005	0.0005	<0.000005	0.0001
PCB-167	2,3',4,4',5,5'-Hexa-	0.00001	0.00001	<0.000005	0.00001
PCB-169	3,3',4,4',5,5'-Hexa-	0.01	0.01	0.00005	0.001
PCB-170	2,2',3,3',4,4',5-Hepta-	0.0001	--	--	--
PCB-180	2,2',3,4,4',5,5'-Hepta-	0.00001	--	--	--
PCB-189	2,3,3',4,4',5,5'-Hepta-	0.0001	0.0001	<0.000005	0.00001

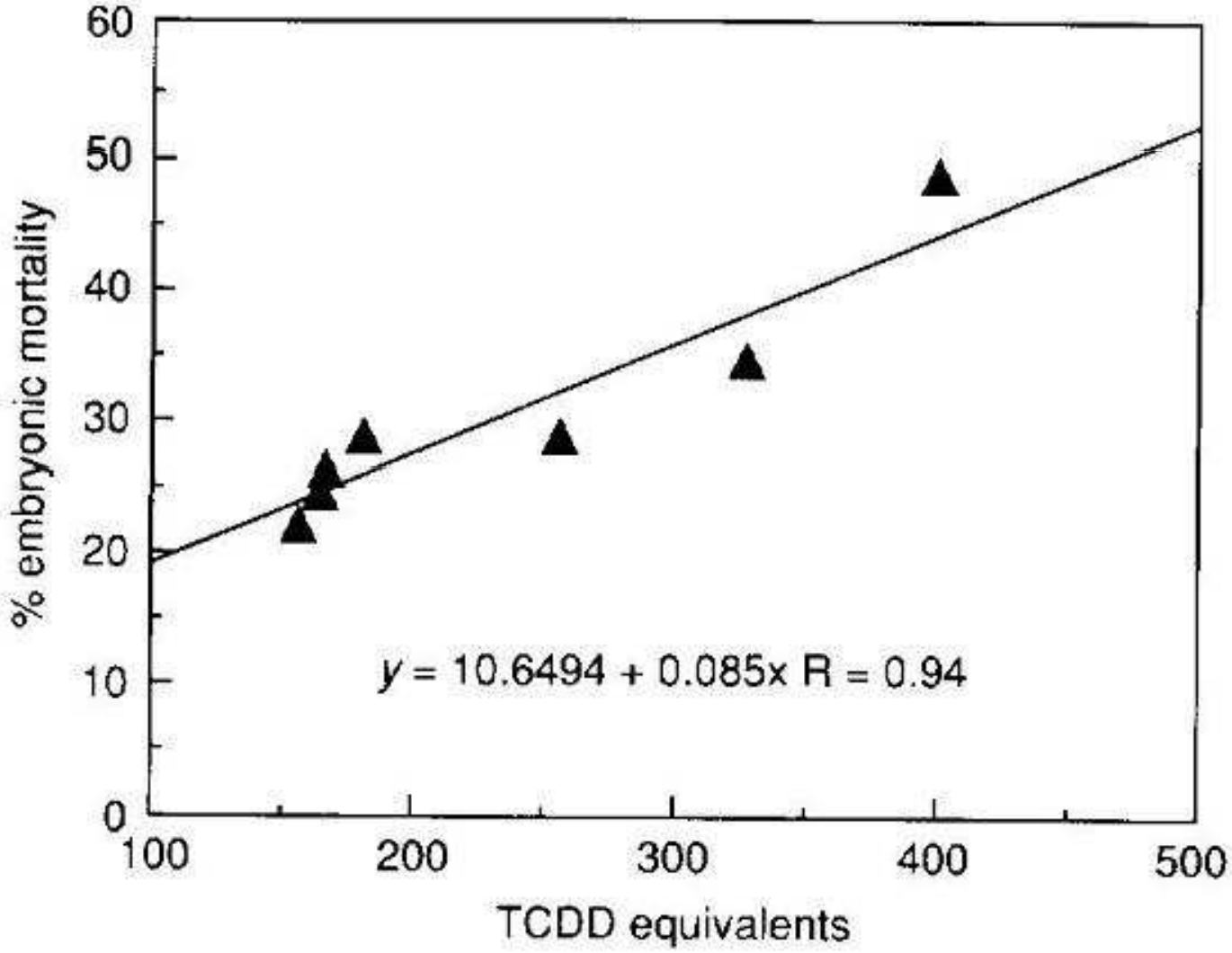


Figure 5.7 Relationship of embryonic mortality of caspian tern and dioxin equivalents. Tillitt *et al.* (1988).

Phase II conjugation enzymes - GSTs

GSTs

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

Substrates reduced GSH + thiol selective probe (CDNB

GST

GSH + CDNB -> GS-CDNB

yellow product (A420), kinetic or endpoint determination

Kinetic assessment

stress -> Induction of GSTs

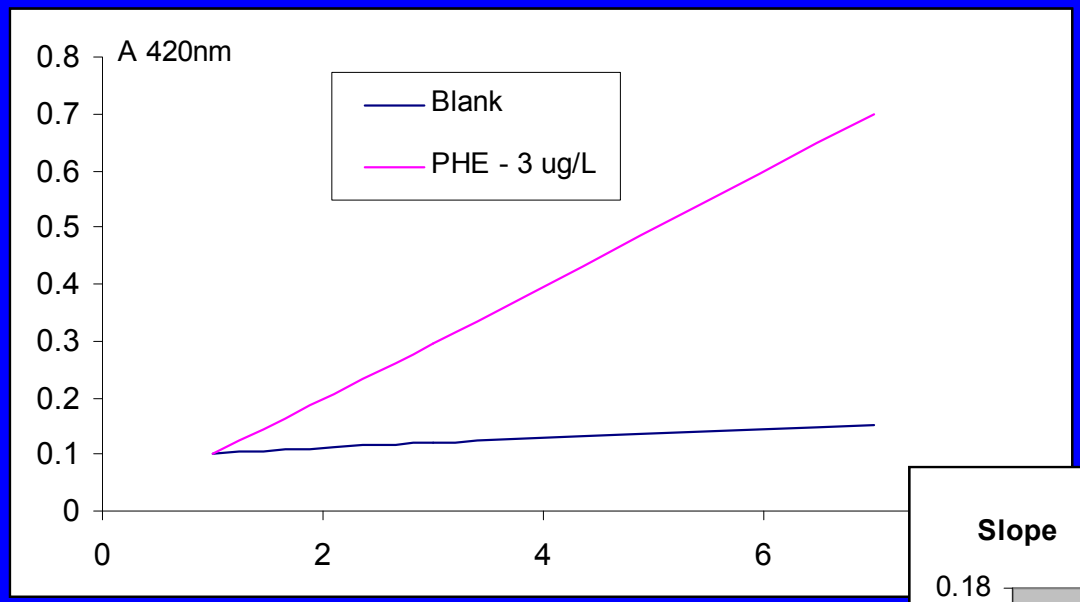
faster reaction -> slope of kinetic increase

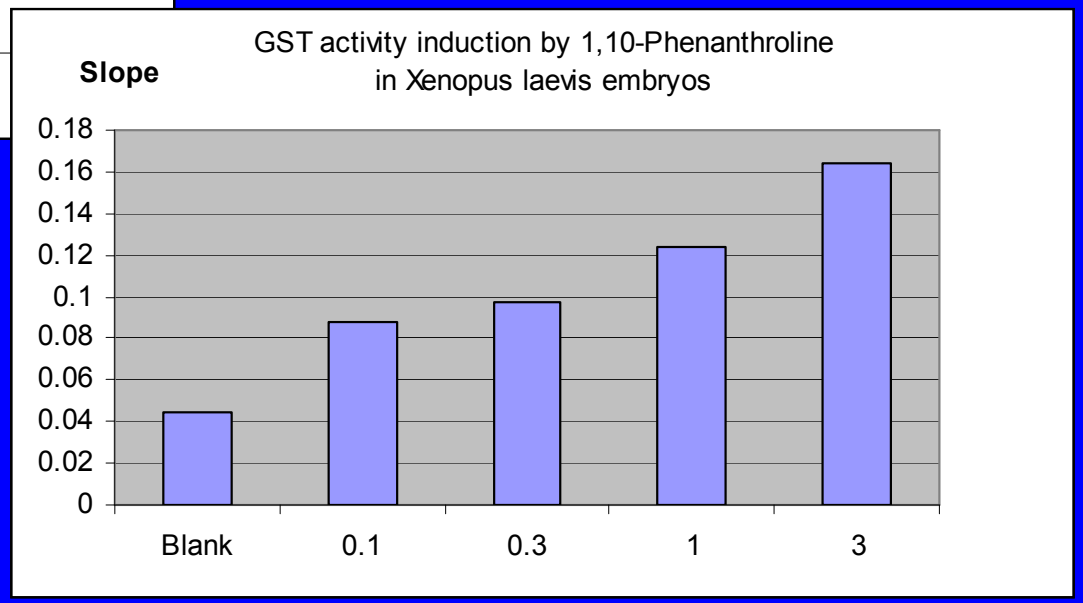
GST activity - example

Kinetic assessment of GSTs

stress -> Induction of GSTs

faster reaction -> slope of kinetic increase





GSH-related oxidative stress enzymes

Glutathion-reductase (GR), Glutathion-peroxidase (GPx)

Enzymatic reactions – differing in substrates (GSH +/- H202 ...)

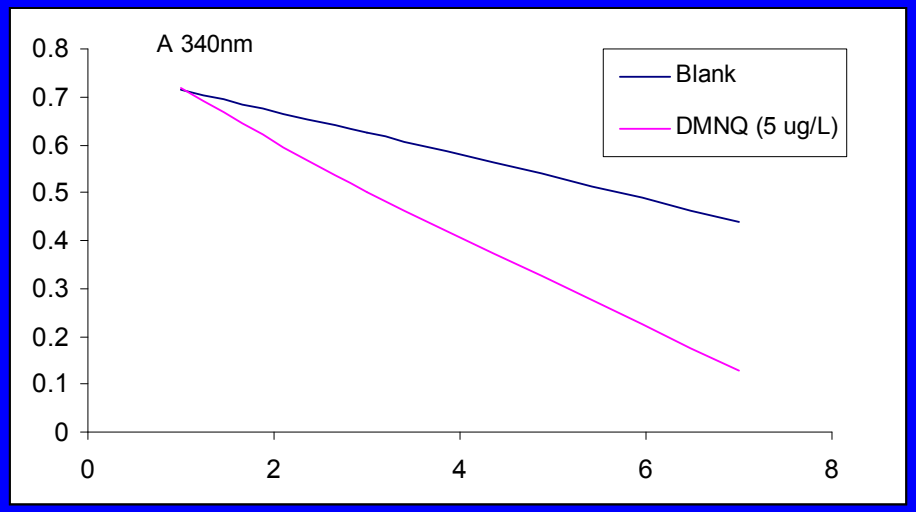
- generally: NADPH consumption during reaction
- NADPH – easily determined (A340 nm)

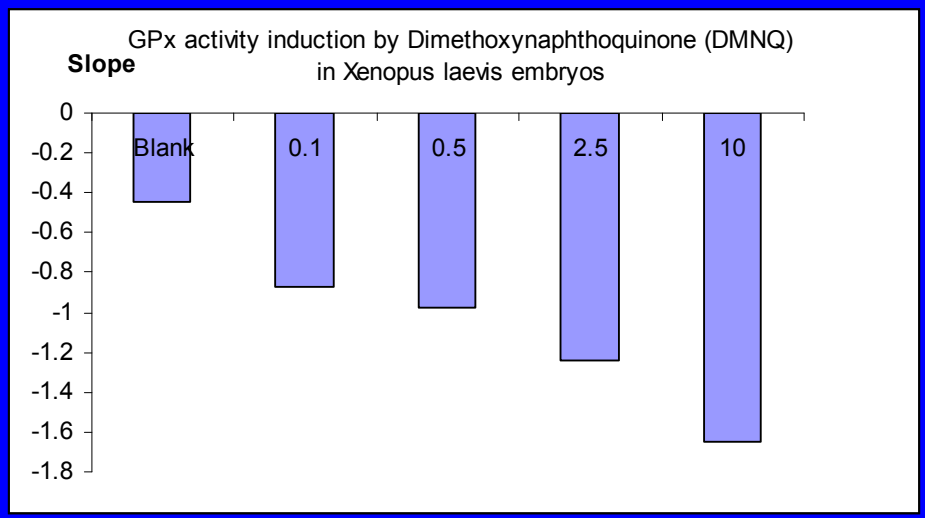
Design – GPx:

Substrates (GSH, organic peroxide, NADPH)

Enzyme (biotic sample)

A340 kinetic record *(slope of kinetics decrease with GPx activity)*





PROTEIN SYNTHESIS

Determination of specific proteins

amount quantification

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

Complementary to enzymatic assays !!!

e.g. CYPs - mRNA -> protein amount -> activity

<u>Examples</u>

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

metalothioneins

Vitellogenin(-like) Vtg proteins in male

Superoxid dismutase (SOD)

Heat Shock Proteins (hsp)

Stress - synthesis of new proteins

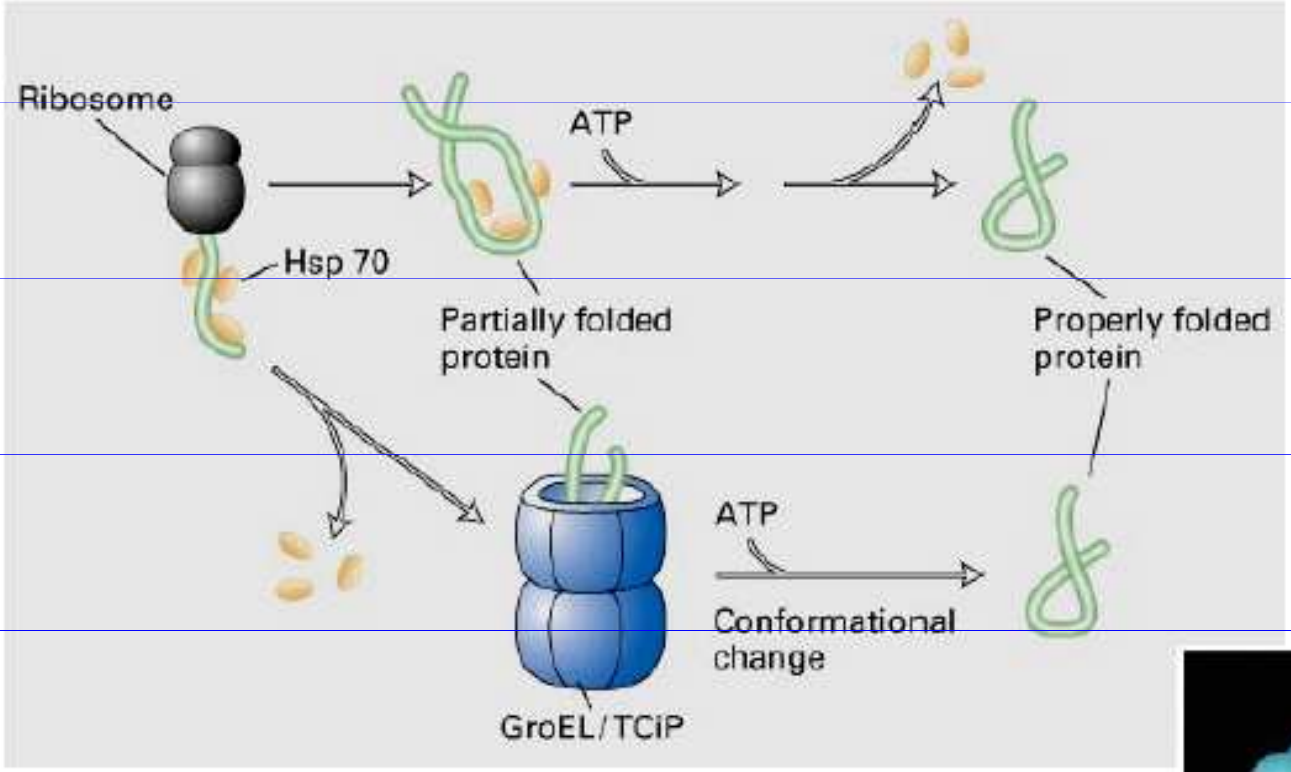
~ equilibrium and homeostasis buffering

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

New proteins must be folded (3D-structure) – „CHAPERONES“

- hsp90, hsp60, hsp 70 – 60-90 kD molecular weight kD
- GENERAL STRESS biomarker, non-specific
- phylogenetically conserved (similar sequences in „all“ organisms)
- **structural similarity => easy determination:**
  electrophoresis + immunoblotting

Heat Shock Proteins (hsp)



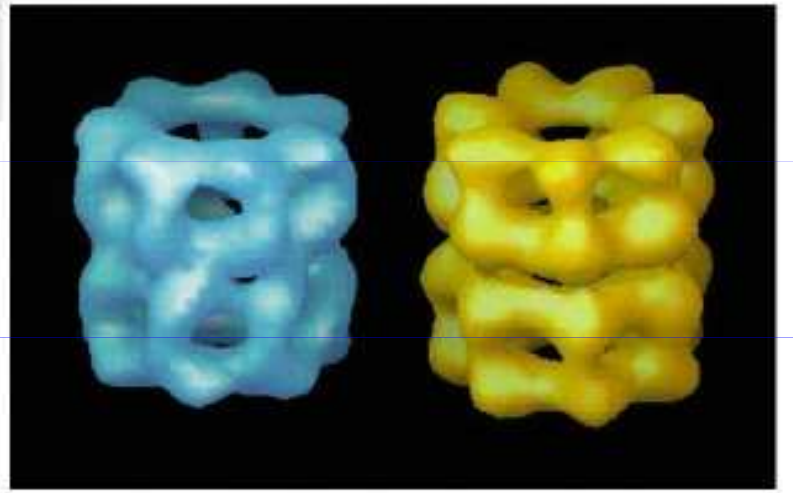
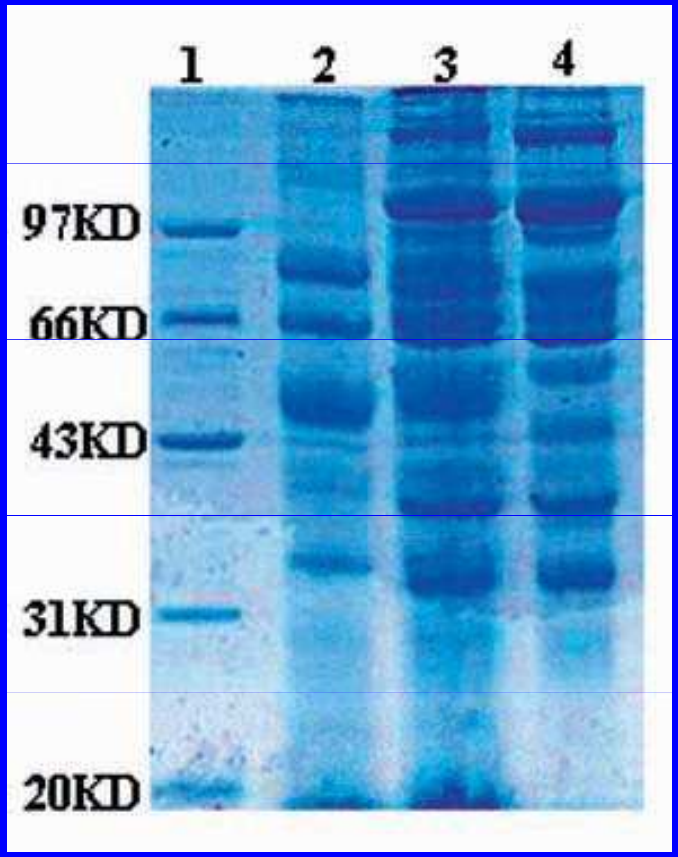
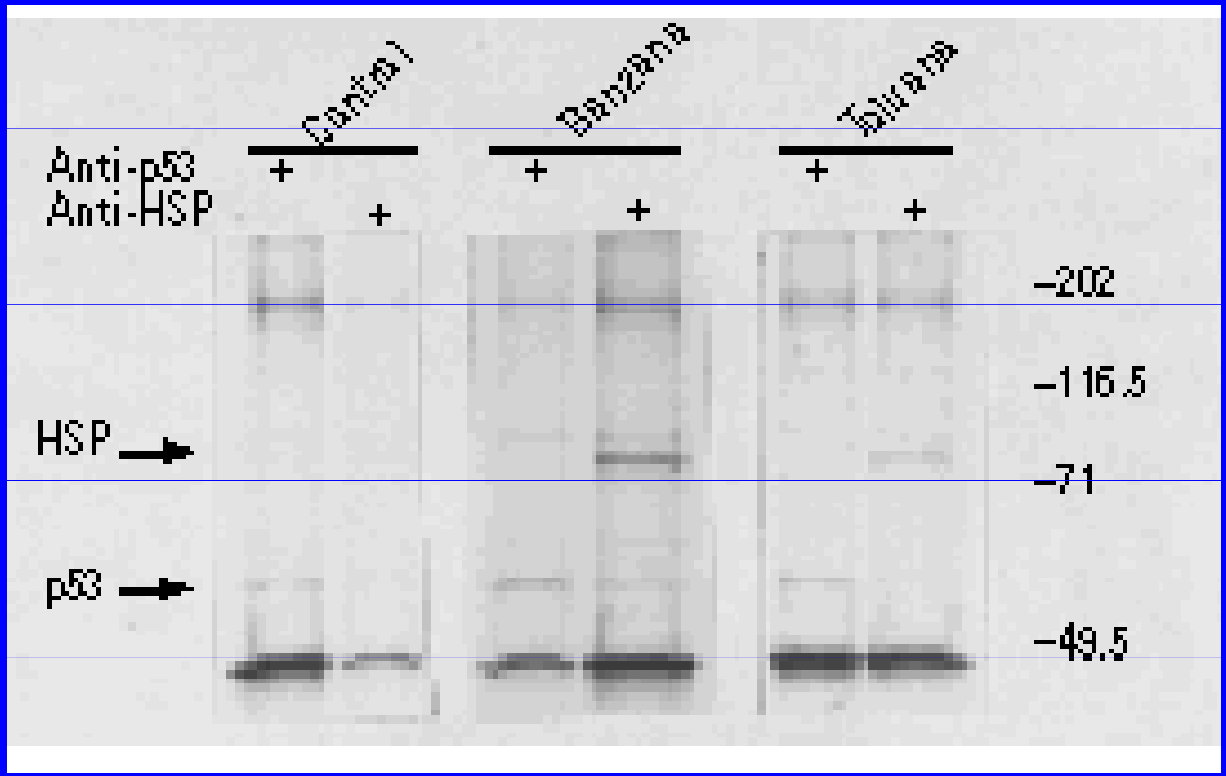


Figure 3-15

HSP determination - example





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

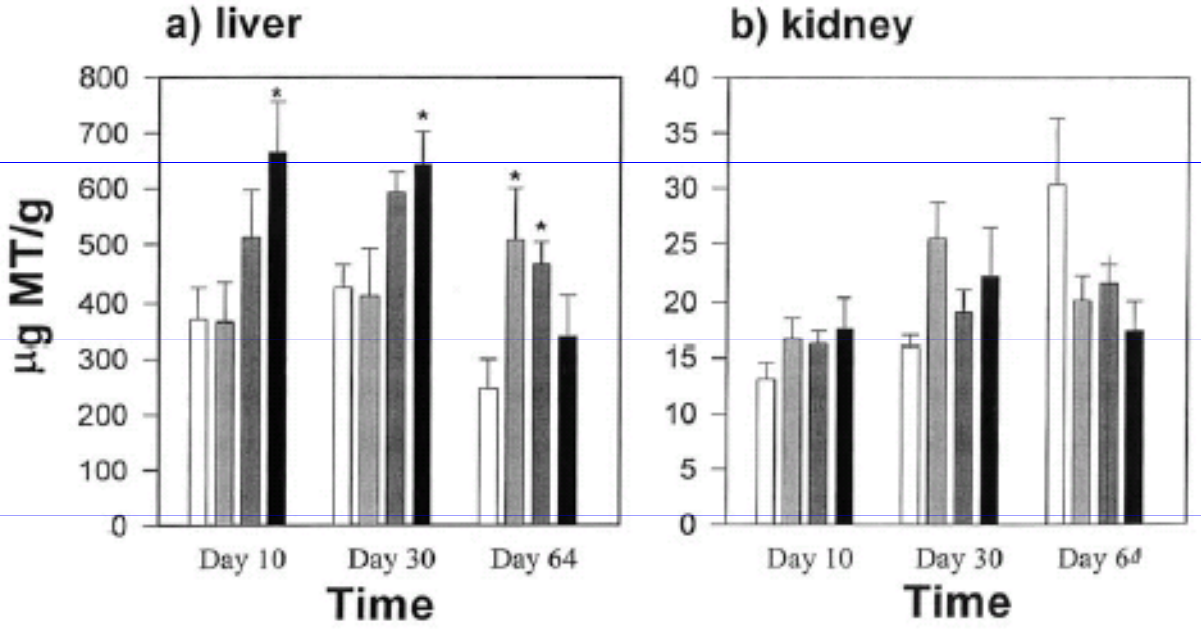


Fig. 2. Metallothionein (MT) concentrations in the (a) livers and (b) kidneys of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (±S.E.). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$).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

Induction of SOD in plants
- protein electrophoresis + immunoblotting

SOD – superoxid dismutase;
induced by oxidative stress

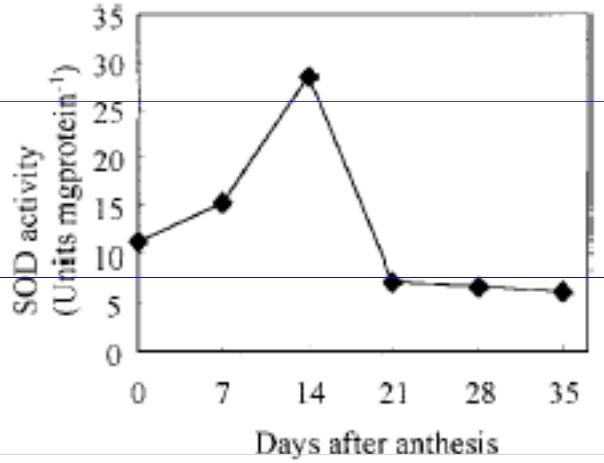


FIG. 1. Effect of monocarpic senescence on the total SOD activity expressed as units mg protein^{-1} in the wheat cv. Kundan. Vertical bars indicate SE (n = 3). In some cases error bars are smaller than the symbols.

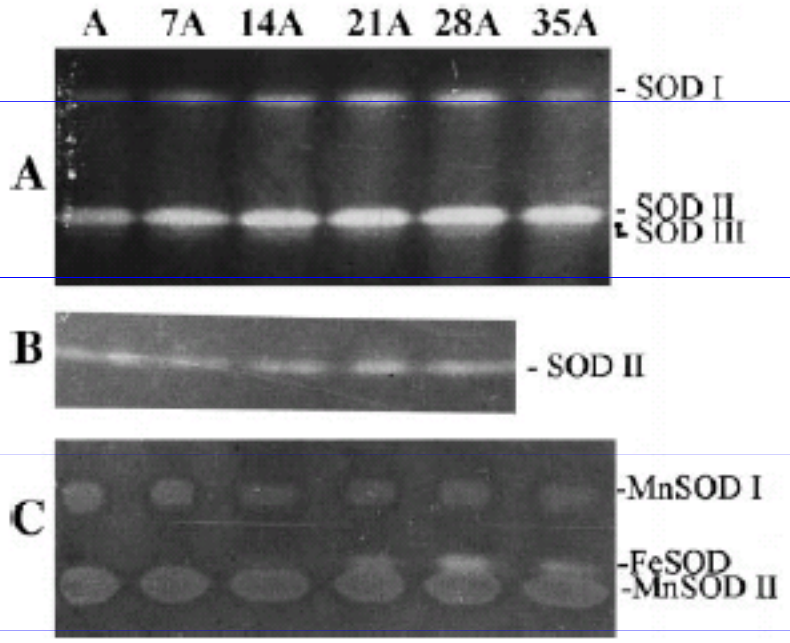


FIG. 2. Separation on nondenaturing activity gels of SOD forms from the leaves of wheat cv. Kundan during monocarpic senescence. In each case 50 μg of protein per lane was loaded. Extracts loaded are from crude samples (A), chloroplasts (B), and mitochondria (C).

bated for 20 min. at 25°C in 50 mM sodium phosphate buffer, pH 7.8, containing either 3 mM KCN or 5 mM H_2O_2. Cu/Zn SODs are inhibited by KCN and H_2O_2, Fe-SODs are resistant to CN^- but inactivated by H_2O_2, and Mn-SODs are resistant to both inhibitors (18). The gels were scanned using a gel documentation system (Bio-

Vitellogenin

Vtg

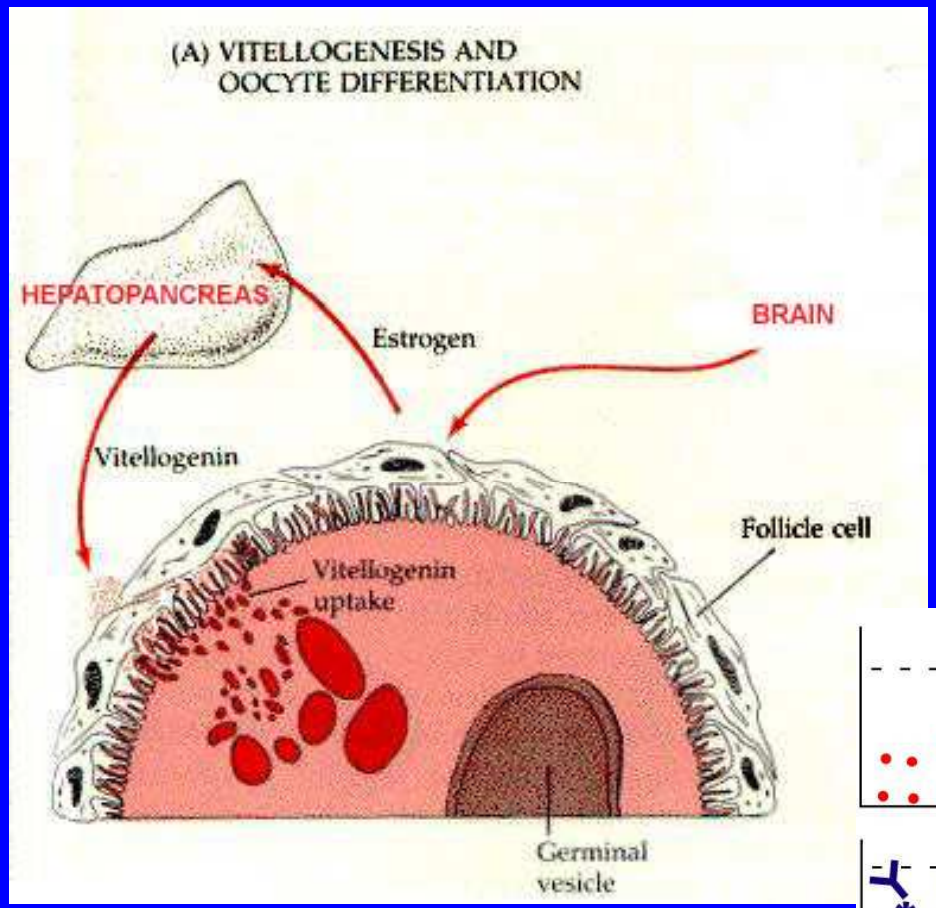
- precursor of yolk proteins, phospho-protein
  -> egg formations (females) at oviparous animals
- synthesised in liver and distributed via blood (haemolymph)
: xenoestrogens & other endocrine disruptors
  -> increased levels or early production in FEMALES
  -> production in MALES

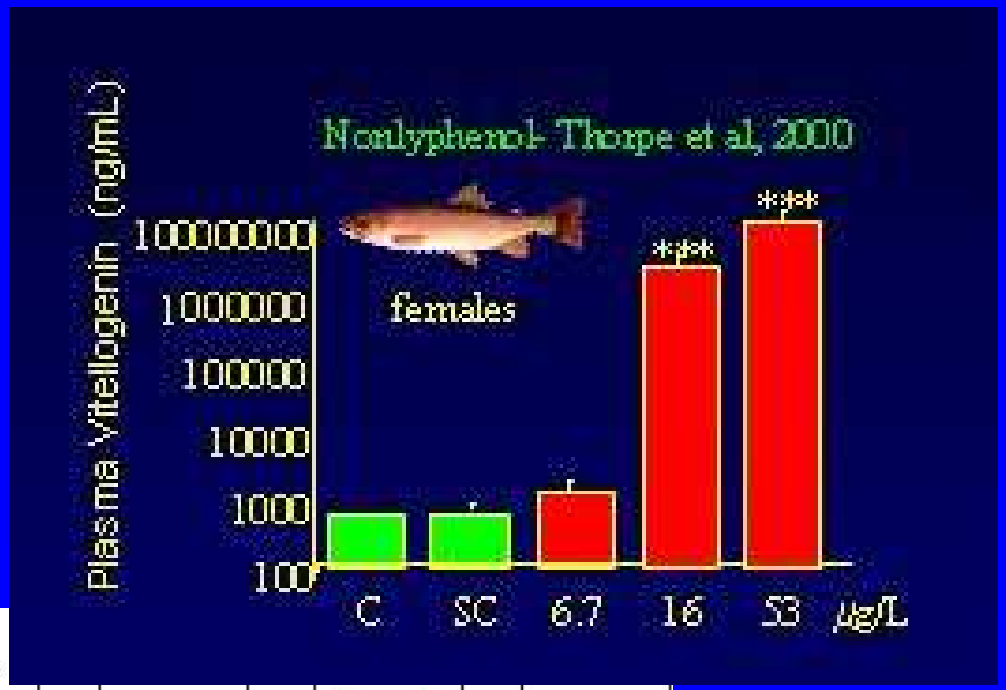
Determination

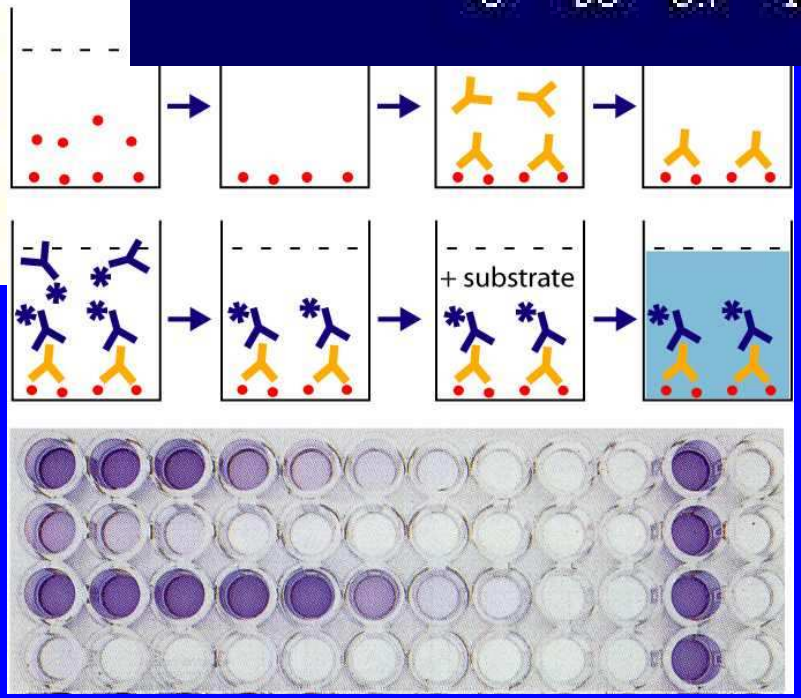
1) ELISA (exposed organisms - F/M, in vitro
  - in vivo - exposed organisms (*biomarker in vivo*)
  - in vitro production in hepatocytes exposed to effluents
  (marker of estrogen-like presence
(-) specific Antibodies necessary for each species (low crossreactivity)

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

Vitellogenin in fish - ELISA







Vitelin-like proteins in mussels

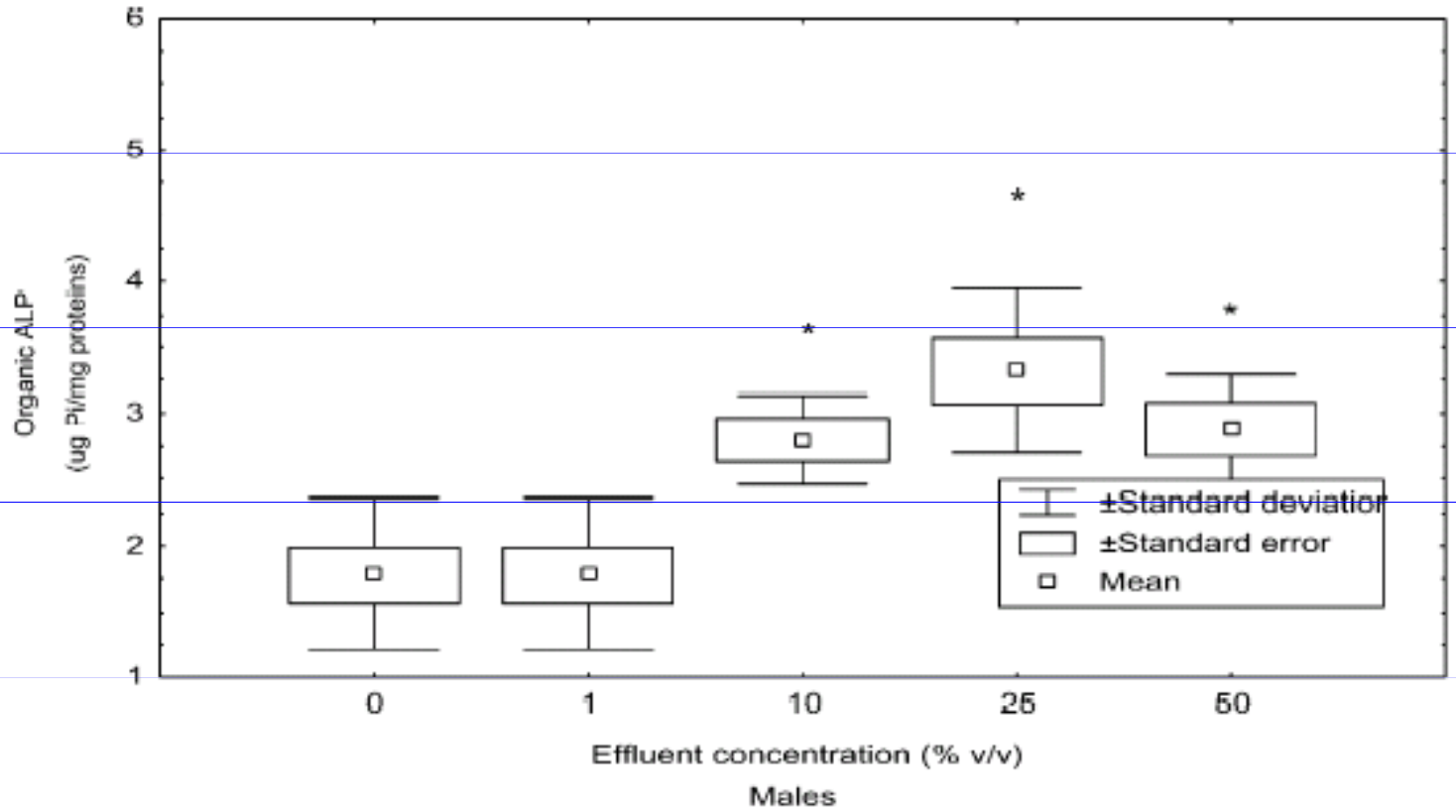


Fig. 4. Induction of Vg by exposure to a municipal effluent. Mussels were exposed for 96 h to a municipal effluent at 15°C. They were then collected for Vg and sex determinations. The asterisk (*) indicates significant difference at $P < 0.05$.

Oxidative stress markers

Several parameters respond to oxidative stress

: enzymes (GPx, GR, GSTs) - *elsewhere*

: antioxidants (GSH, vit E)

: markers of oxidative damage (MDA, 8OH-dG)

Determination of GSH (complex role in organism)

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

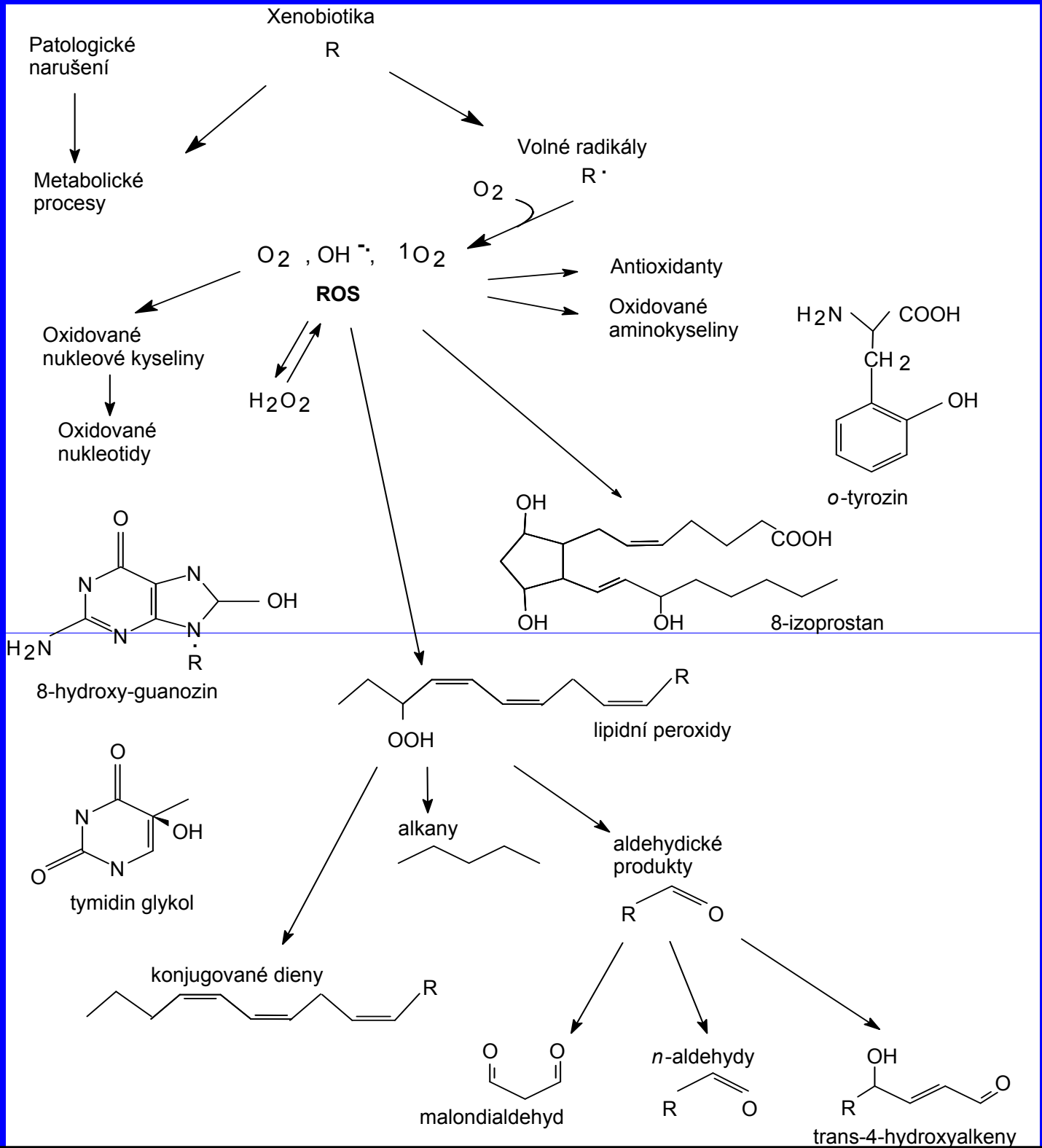
Total glutathione = reduced GSH + oxidized GSSG

GSH + Ellman´s reagent (DTNB) -> Reduced GSH

GSH + Glut.Reductase + DTNB -> Total GSH

Total – Reduced = Oxidized

Oxidative stress markers



Malonyldialdehyde (MDA)

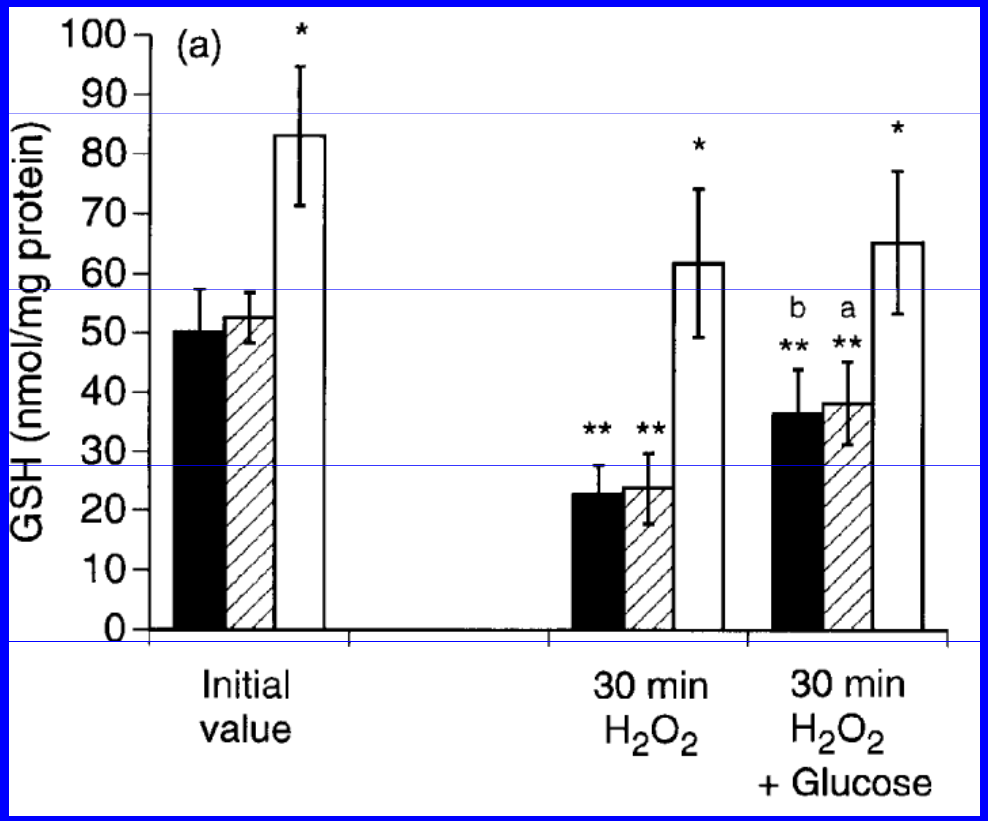
MDA – formed from oxidized membrane phospholipids
: determination: HPLC or TBARS method

TBARS – ThioBarbituric Acid Reactive Species
: less specific than HPLC (+/- aldehydes)
: easy determination (spectrophotometry)

Method:

1) sample extract (virtually containing MDA) + TBA
3) boiling (cca 30´ / 90°C)
=> formation of red/violet coloured product
4) determination by spectrophotometry (A 540 nm)

GSH & MDA - modulation / - example



patients control