

# Biomarkers

## Biomarkers

- markers in biological systems with a sufficiently 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 (health, toxicology and 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 testing to characterize potencies of xenobiotic to induce specific biological activity (genotoxicity, estrogenicity, dioxin-like activity, tumor promotion ...)*

*= biological potencies (markers) of potential hazards*

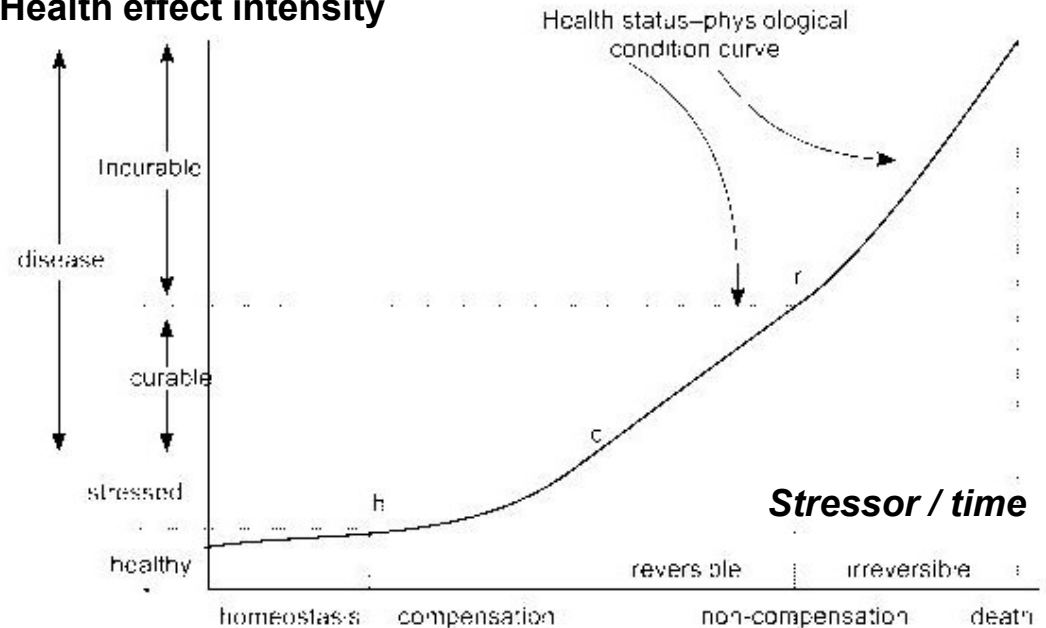
# Biomarkers & Exposure

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

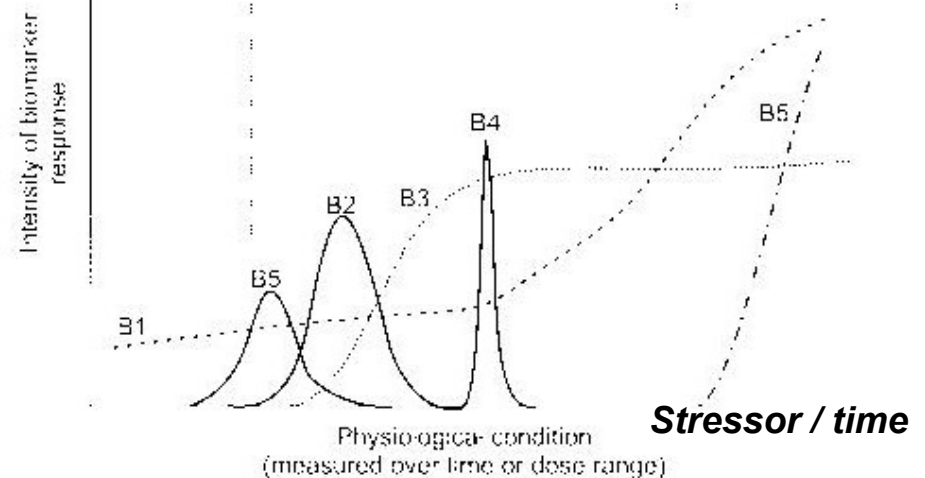
## Biomarkers:

- temporal change
  - B5, B2; short period: B4
- continuous increase - B3
- repeated occurrence (B5)
  - irreversible change

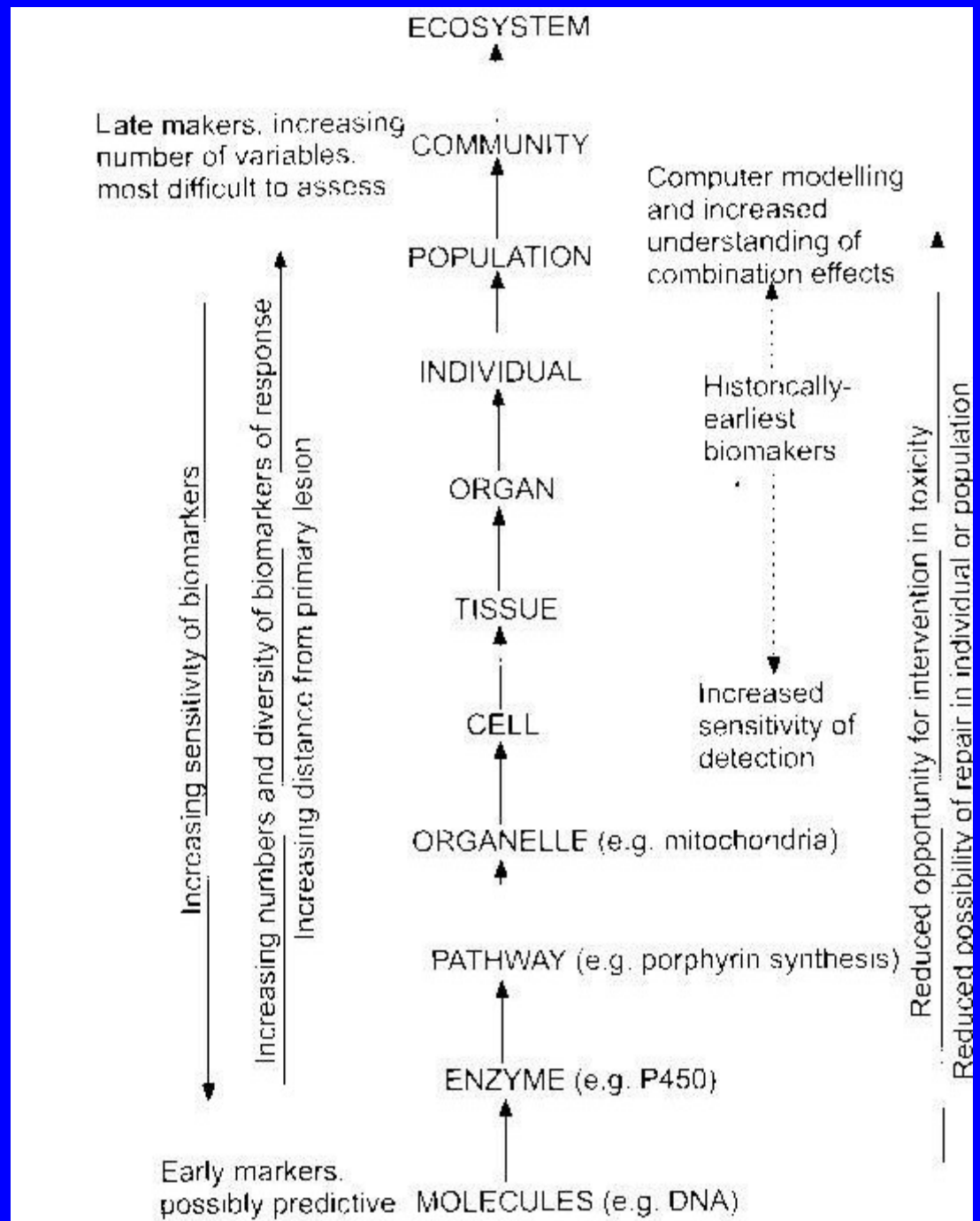
## Health effect intensity



## Biomarker intensity



# Biomarkers at different levels of biological organisation



# Biomarkers - classification

## Categorization US National Academy of Sciences

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

## Continuum exists among biomarkers

example: adducts of toxicant with DNA

? *biomarker of exposure* / ? *response*

## **Specific (selective) *in vivo* biomarkers**

- Biomarkers selectively reflecting specific types (mechanisms) of toxicity
- *E.g. inhibition of AcCholE :  
exposure = organophosphates; effect = neurotoxicity*
- + provides specific information
- multiple biomarkers must be measured in parallel

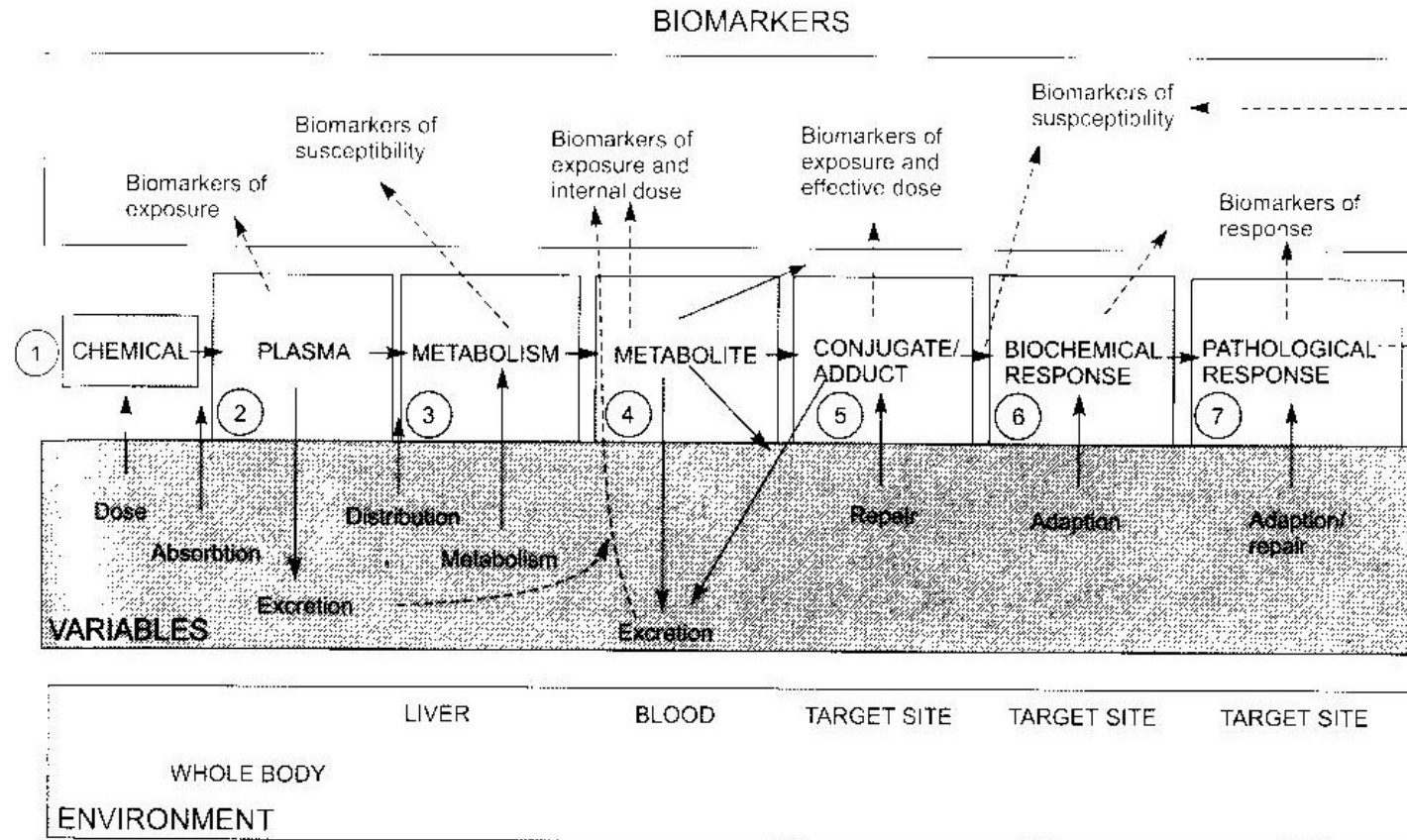
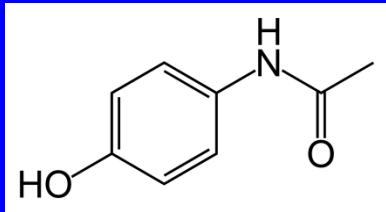
## **Non-specific (non-selective) *in vivo* biomarkers**

- 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 - sampling

- Non-destructive (non-invasive)
  - : blood / haemolymph collection & analyses
  - : skin, feather, hair ...
  - : *life of the organism not affected*
- Destructive (invasive)
  - : whole animal -> multiple biomarker evaluation

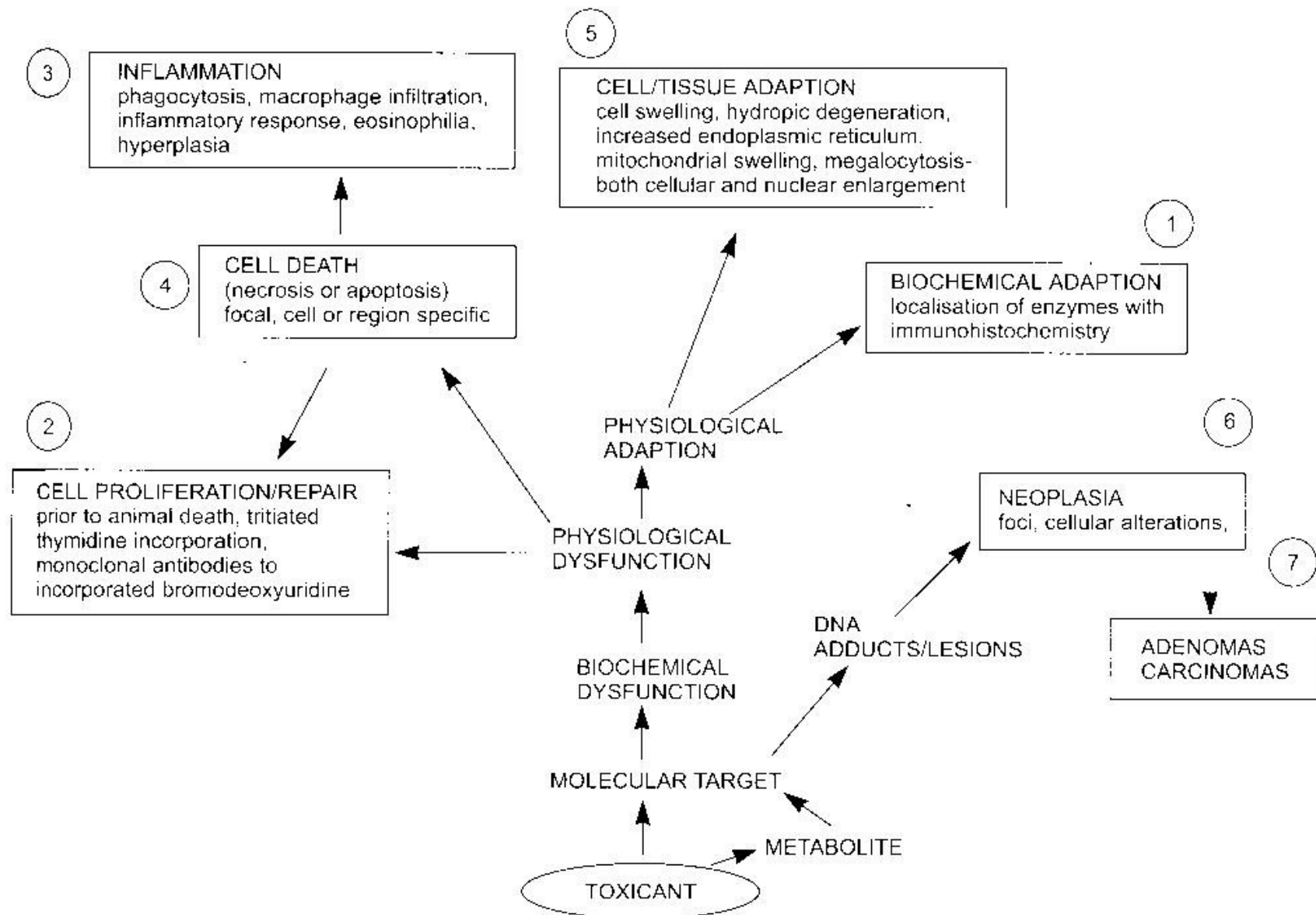
# EXAMPLE - Paracetamol



- (1) paracetamol
- (2) parent compound measurement - biomarker of 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 inflammation 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- <i>O</i> <sup>6</sup> guanine	Styrene exposure	
	Protein adduct	N <sup>7</sup> -Guanyl-aflatoxin B <sub>1</sub>	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	
		ALP (alkaline phosphatase)	Bile duct toxins	
		CK or CPK (creatine kinase)	Heart/muscle toxins	
		Serum/plasma biochemistry	Urea (changes)	Hepatotoxic and nephrotoxic compounds
			Protein (reduced, e.g. albumin)	Hepatotoxic compounds
			Bilirubin	Liver injury
			Prothrombin	Warfarin (rodenticide)
			Glucose, raised creatinine, GSH conjugates	Pancreatic abnormalities, kidney damage
	Liver glutathione		Reactive oxygen species	
	P450 induction		Polycyclic aromatic hydrocarbons	
	Clotting time	hsp 60, hsp 70, hsp90	Cadmium, heat	
		Metallothionein	Heavy metals, e.g. cadmium	
		Antibodies, e.g. IgG	Antigens	
		Dermatitis	Nickel	
		Chromosomal aberrations, micronuclei	Genotoxic agents	
Heart rate, temperature, sleeping time		Barbiturates		
Breeding patterns, migrations		Climate change		
Susceptibility	Phenotype	Acetylator phenotype ( <i>NAT 2</i> )	-	
	Oncogenes	Dominant oncogenes ( <i>ras, mic</i> )	-	
		Recessive suppressor gene ( <i>p52</i> )	-	
	'Cancer' genes	Breast-ovary cancer gene ( <i>BRCA 1</i> )	-	

# Further examples

## Toxicity 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 <sub>3</sub> and T <sub>4</sub> are sensitive
Retinols	+	Liver	Advances to use plasma are being made
Porphyryns	+?	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 complex exposures/contamination ?**

- integrated approach needed

- nonspecific biomarkers (*hsp*) ...

# Multiple biomarker evaluation

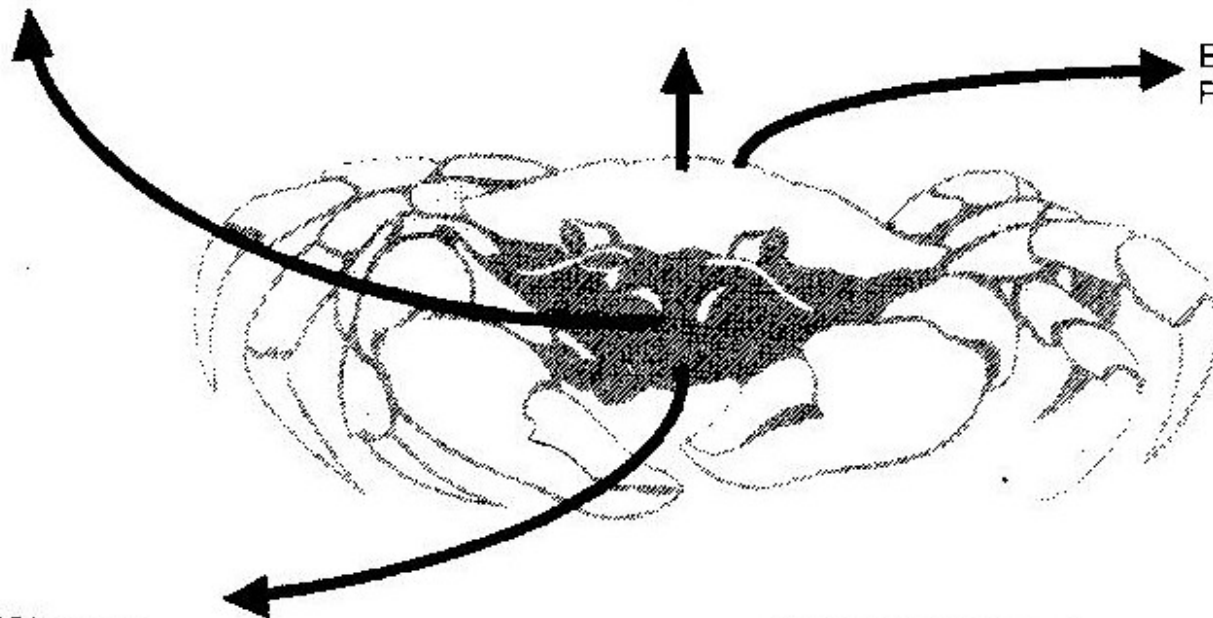
## GILLS

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

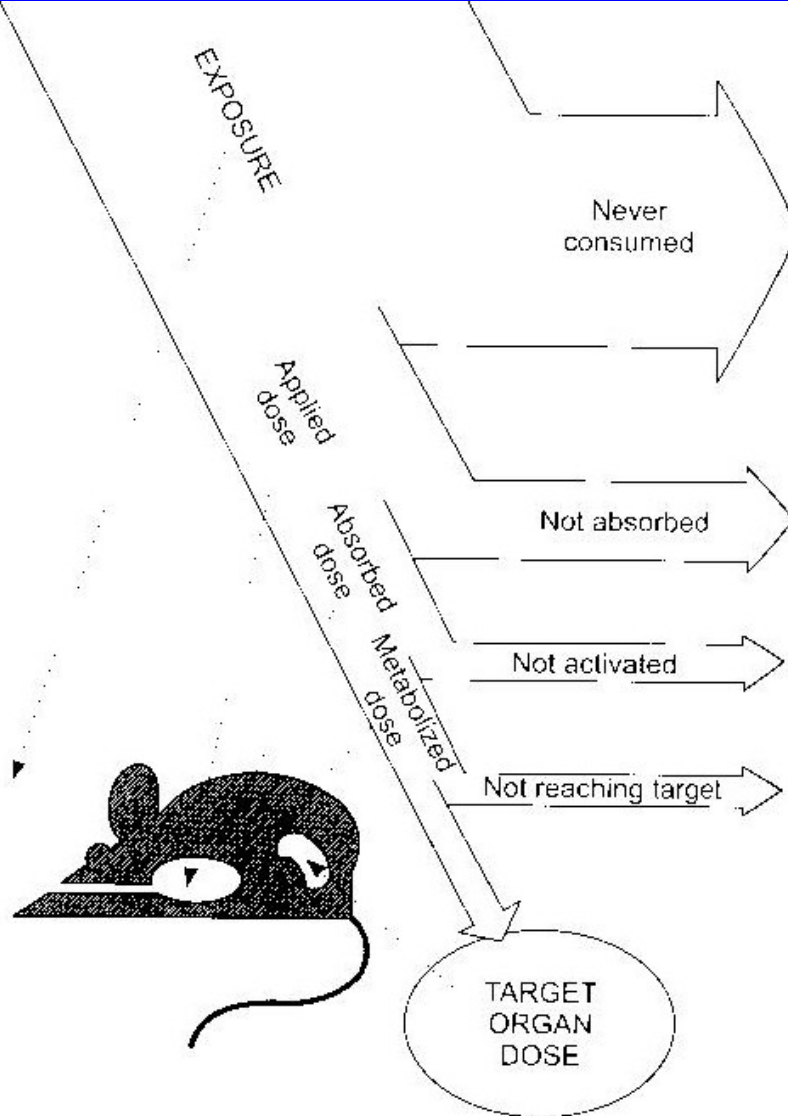
Acetylcholinesterase activity  
Butyrylcholinesterase activity  
Micronuclei  
Total proteins

Hepatopancreas and  
gills as % of body weight

# Biomarkers of susceptibility

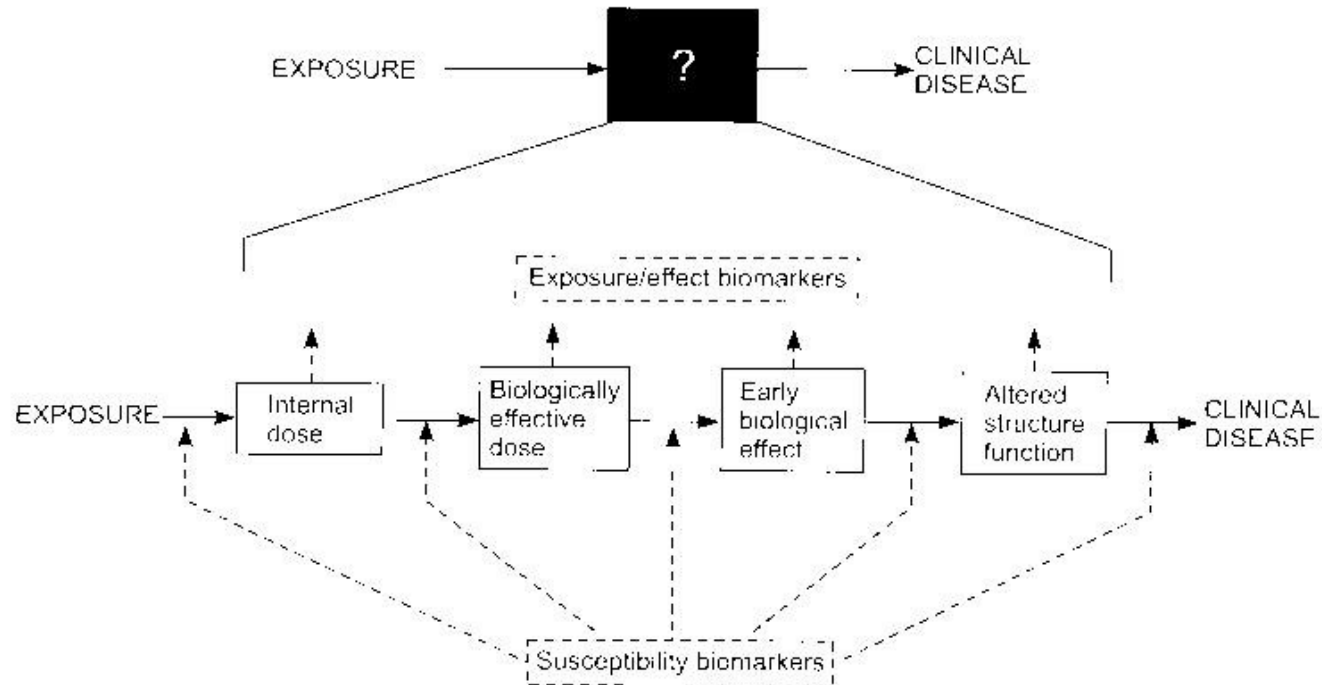


# Toxicokinetics & Biomarkers of susceptibility



**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.

# 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.



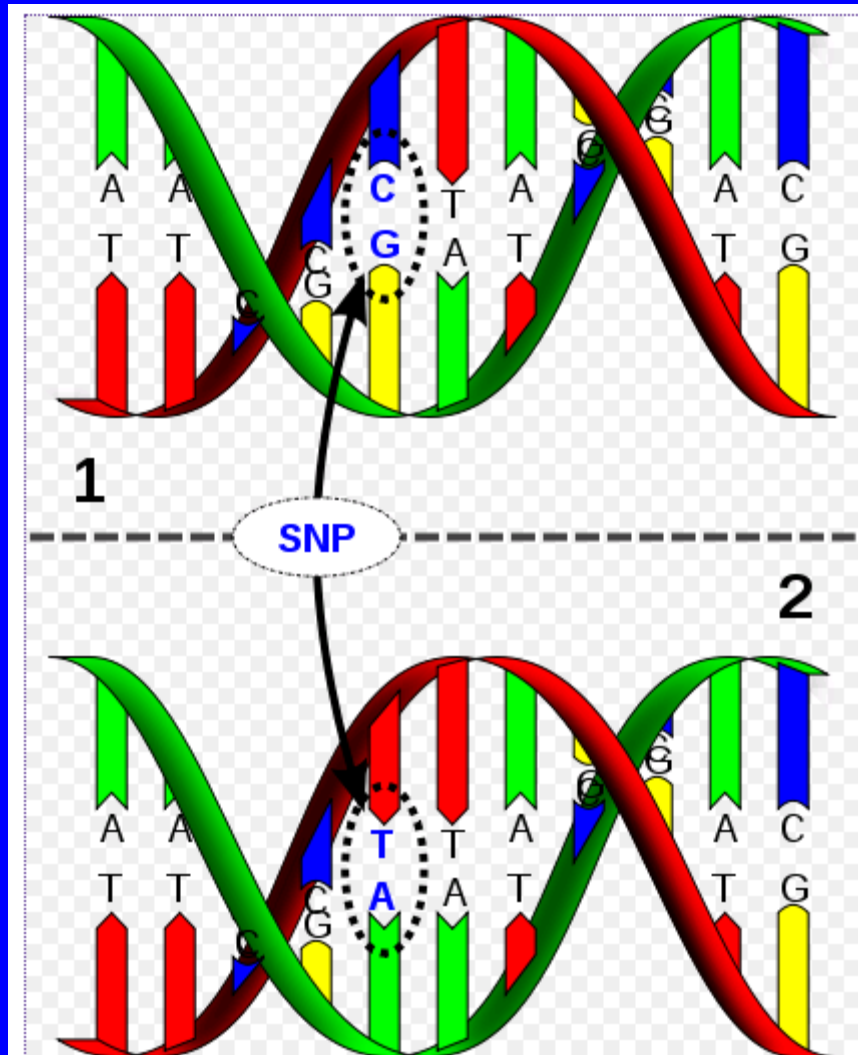
# Biomarkers of susceptibility

## Metabolism and genotype

- genetic polymorphism in detoxification enzymes
- variability in specific isoenzymes
- susceptibility to „activate“ toxicants:  
*example: N-acetylation of arylamines – NAT2*
- familial cancers
- susceptibility to genotoxins
- susceptibility to drugs (including anticancer drugs)

# Example: genetic polymorphism

## SNPs - single nucleotide polymorphism

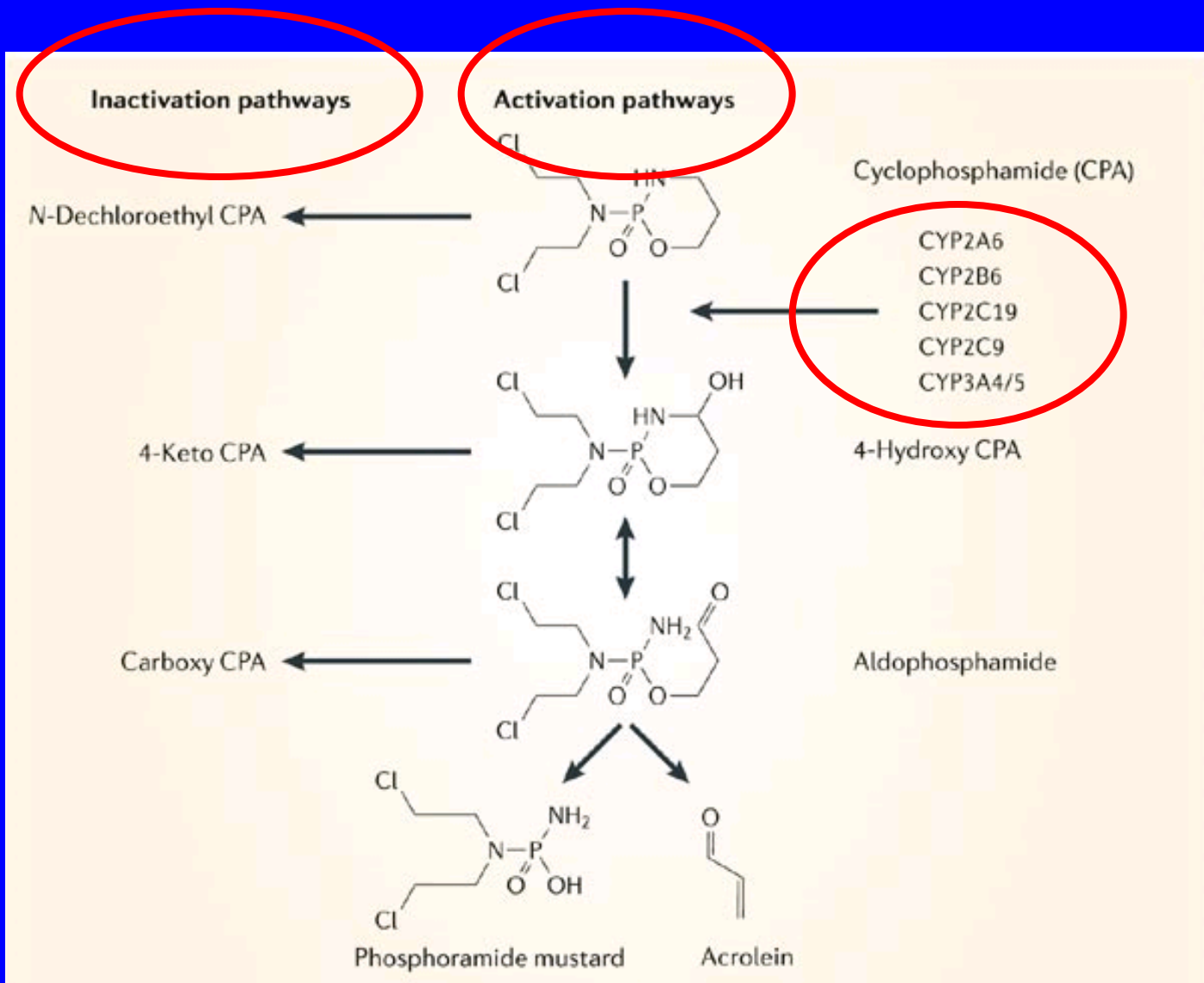


SNP -> affects protein functions

Many genotypes (from many individuals) must be sequenced to identify SNPs

(Some) SNPs identified for some (few) genes

# Example: cyclophosphamide toxicity



**Genetic polymorphism**

# Example: genetic polymorphism

## CYP450 Enzymes and Polymorphisms



Diagnostics

Enzyme	Fraction of drug metabolism	Major polymorphisms
CYP3A4	40-45%	Rare
CYP2D6	20-30%	*2xn, *4, *10, *17, *41
CYP2C9	10%	*2, *3
CYP2C19	5%	*2, *3
CYP1A2	5%	*1K
CYP2B6	2-4%	-
CYP2E1	2-4%	-
CYP2A6	2%	*4, *9
CYP2C8	1%	*3
CYP3A5	<1%	*3

Alleles known to be involved in polymorphism

The CYP 2D6 gene is extremely polymorphic with more than 70 allelic variants described so far <sup>1</sup>

# **Biomarkers of EXPOSURE**

# Biomarkers of Exposure

## Biomarkers of ... *internal / effective dose*

*depending on toxicokinetics*

### - internal dose (short / long term)

– *Cd in urine, DDE in fat tissues*

- should be easy to sample (urine, breath)

- instrumental analytical methods (analyses of toxicant)

### - effective dose

- the chemical interacted with the biological target

= ADDUCTS

# TOXICANT ADDUCTS with BIOMOLECULES

## 1) Selective adducts (chemical-specific)

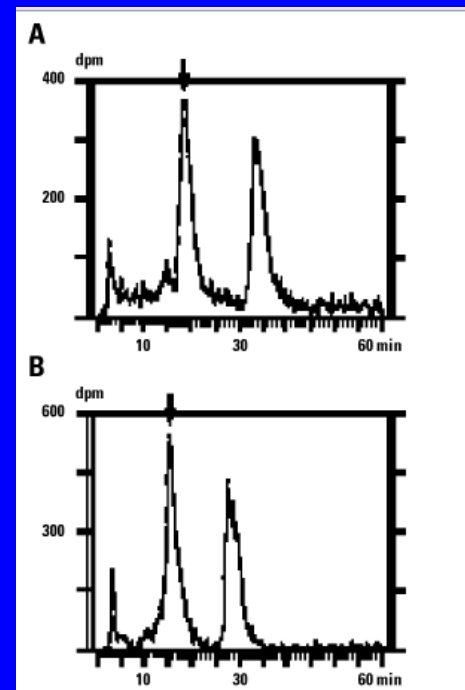
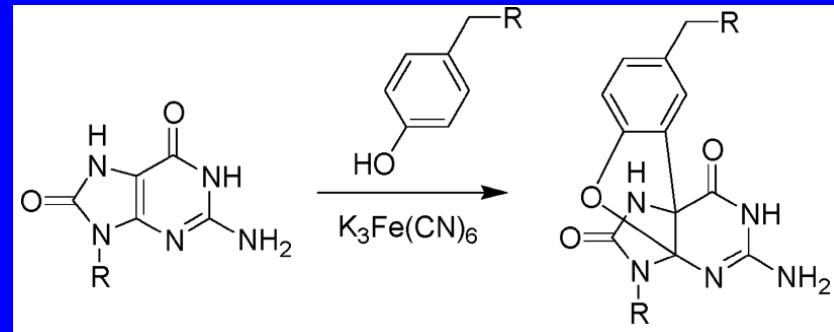
- *DNA aducts:*

*styrene-oxide-O6-guanine*;  
*N7-guanyl-aflatoxin B1*;

- *hemoglobin-pesticides*

- extraction  
and chemical determination (HPLC, GC)

## 2) Non-selective adducts



**Table 1** Reported human haemoglobin adduct levels for various xenobiotics

Chemical (type of exposure)	Adduct/analyte	Method	Adduct level (nmol g <sup>-1</sup> haemoglobin)
<i>N,N</i> -Dimethylformamide (occupational)	3-Methyl-5-isopropylhydantoin	Hydrolysis; GC-MS	75-1000 (exposed) 4-12 (control)
Epichlorohydrin (occupational)	<i>N</i> -(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- <i>S</i> -yl)acetaminophen	Immunoassay	100-4100
PAHs (occupational)	BPDF-Hb	Spectrofluorimetry	0.005-0.139
Ethylene oxide (occupational)	<i>N</i> -Hydroxyethylvaline	Modified Edman; GC-MS	5-20 (exposed) 0.1-0.5 (control smokers) 0.01-0.1 (control non-smokers)
Ethene (occupational)	<i>N</i> -Hydroxyethylvaline	Modified Edman; GC-MS	0.02
Propylene oxide (occupational)	<i>N</i> -Hydroxypropylvaline	Modified Edman; GC-MS	0.05-3.5 (exposed) < 0.02 (unexposed)
Acrylonitrile (smoking)	<i>N</i> -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)	<i>N</i> -(2-Carbamoyl)ethylvaline	Modified Edman; GC-MS	9.5 (production workers) 0.054 (laboratory workers) 0.116 (smokers) 0.031 (non-smokers)
Butadiene (occupational)	<i>N</i> -(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)



# TOXICANT ADDUCTS with BIOMOLECULES

## 2) Non-selective adducts

– binding with DNA (*proteins*) but no further information on the structure of adduct (*causative agent*)

### - Analysis:

- *<sup>32</sup>P-postlabelling assay*

- *DNA-strand breaks*

- *comet assay*

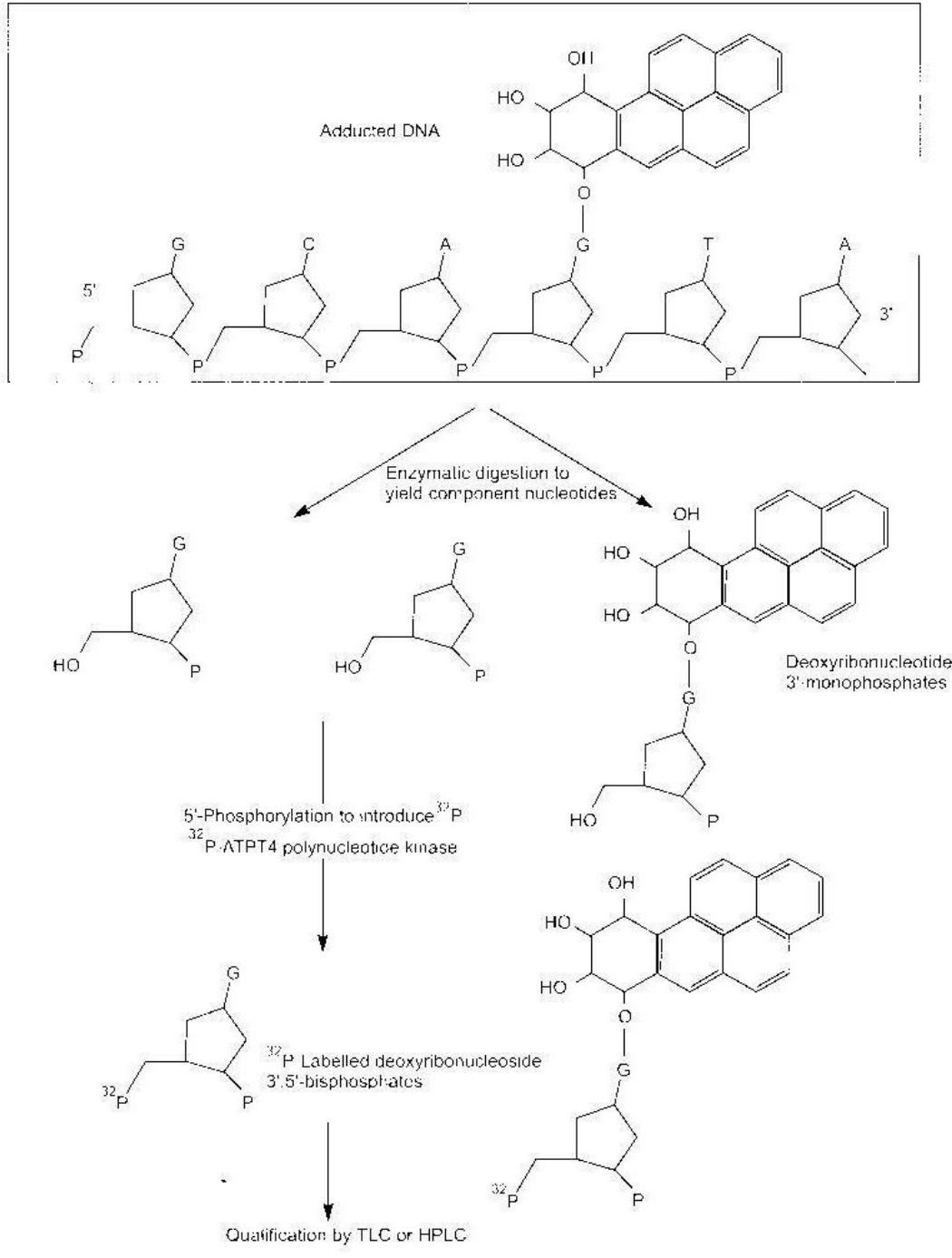
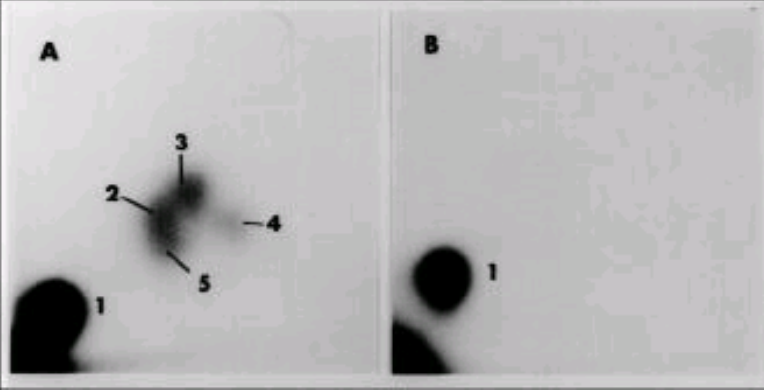
- *identification of oxy-DNA*

*8-hydroxy-2'-deoxyguanosine*

# 32P-postlabelling assay

## TLC result

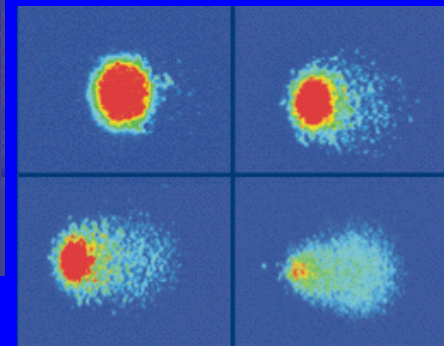
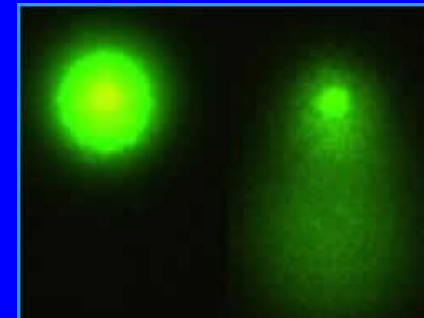
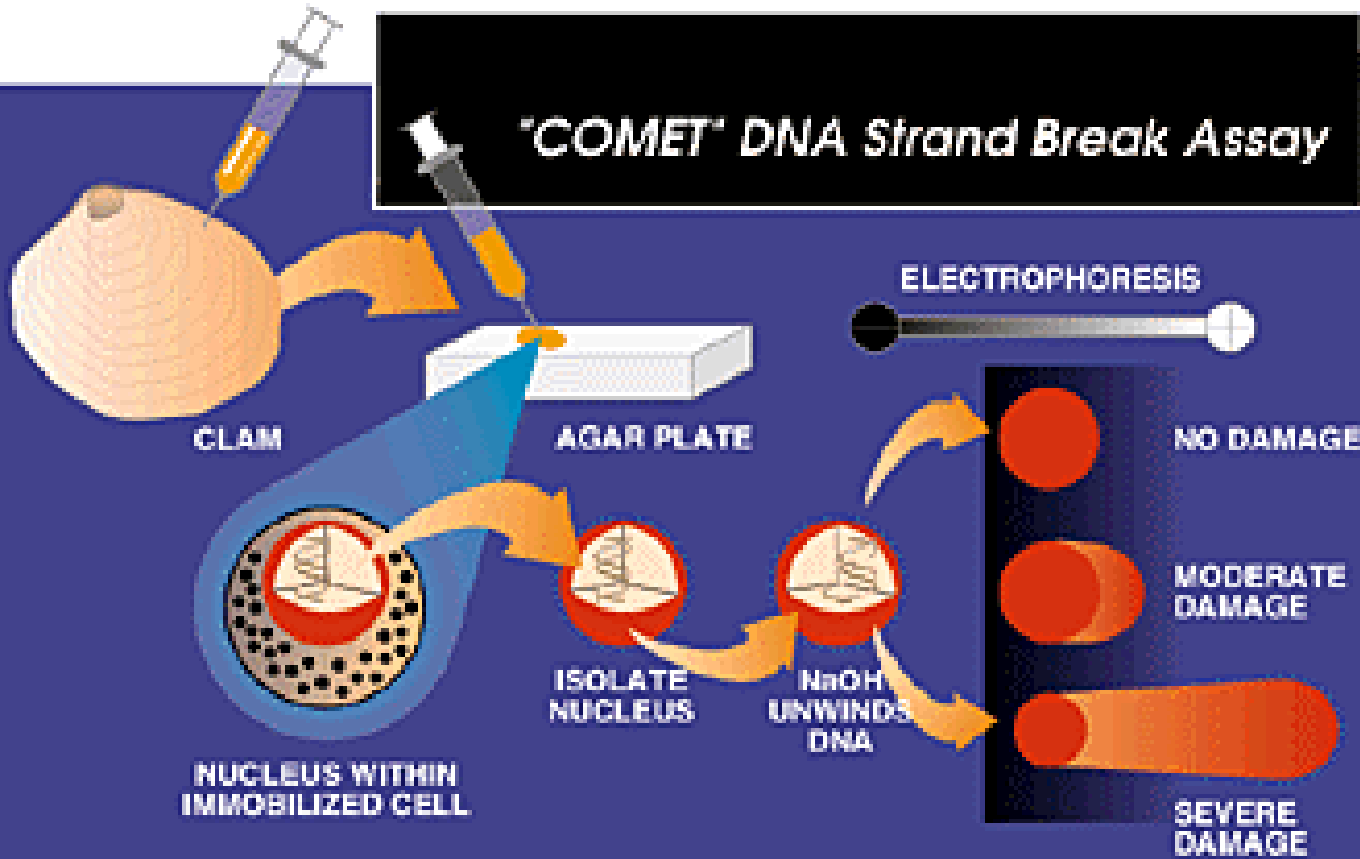
A - 2-5 = various adducts  
 B - controls



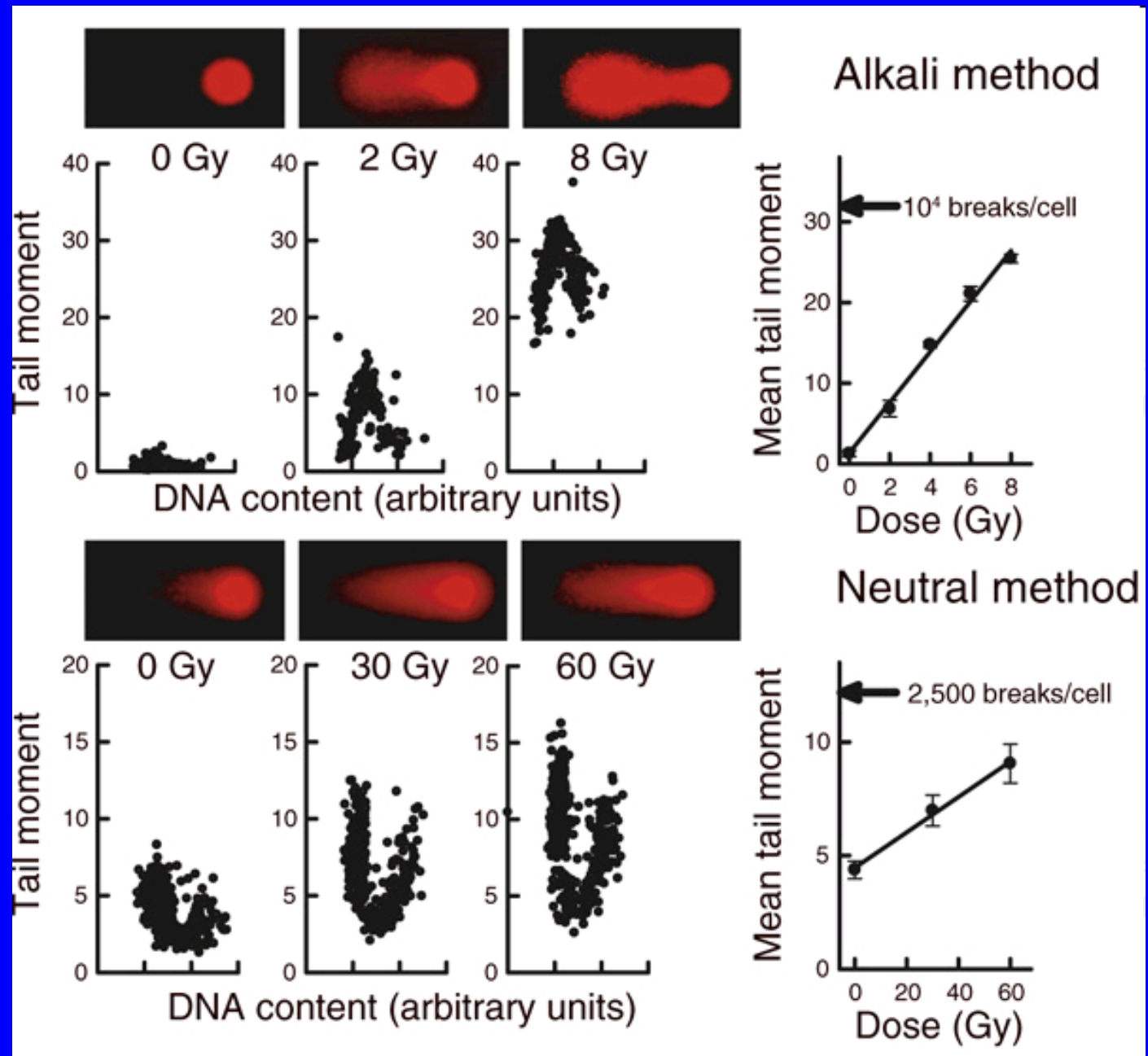
# Comet assay



*"COMET" DNA Strand Break Assay*



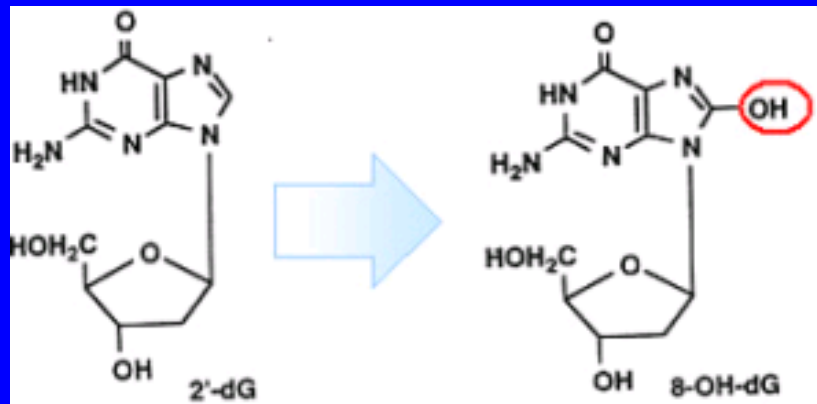
# Example results - Comet assay vs. radiation



# 8-hydroxy-2'-deoxyguanosine analysis

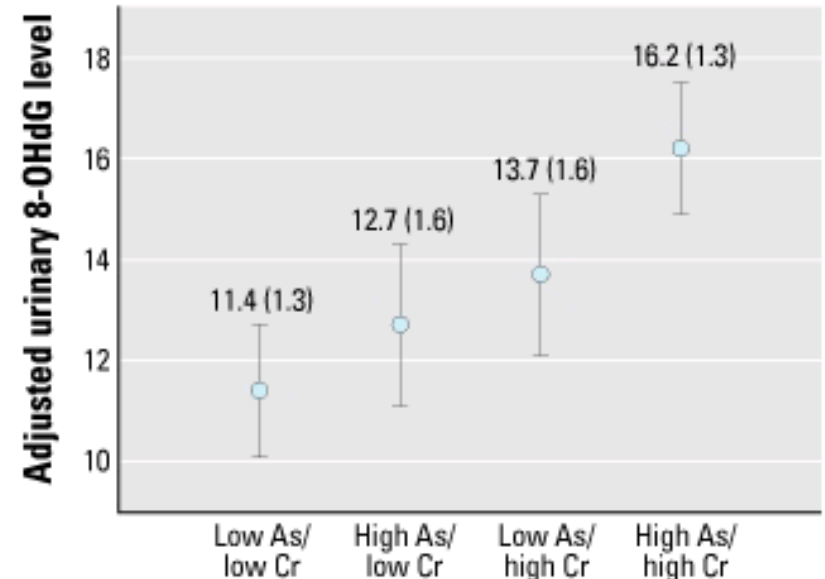
## Oxidative damage to DNA

- many causes
- 8-OH-dG is the most common DNA marker



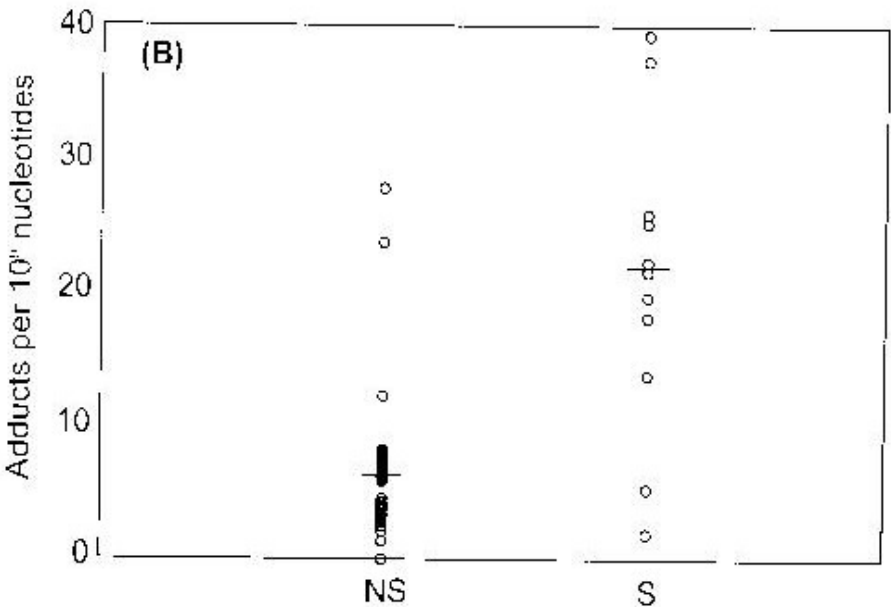
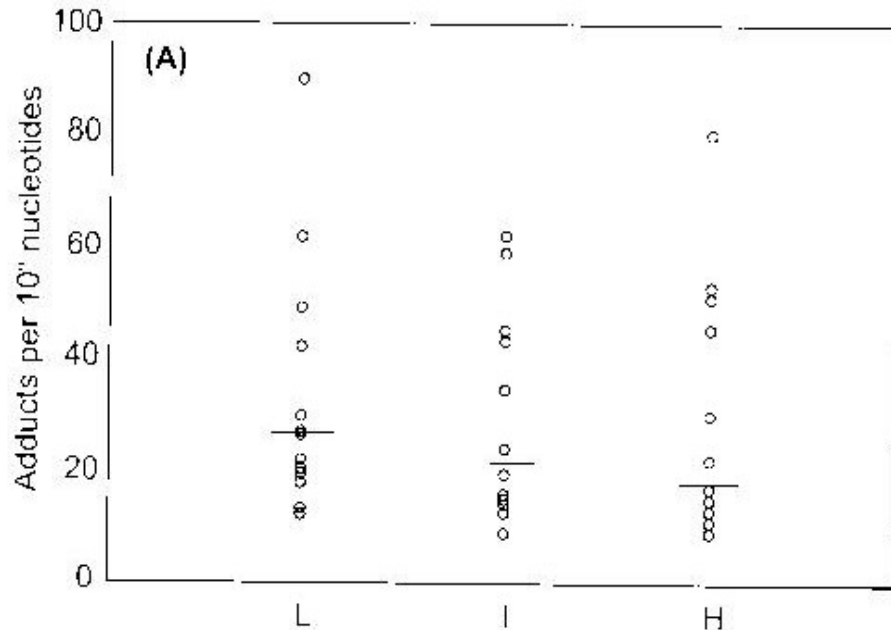
## Analysis:

- HPLC
- immunochemistry (ELISA)



**Figure 1.** Adjusted urinary 8-OHdG level (ng/mg creatinine) by urinary arsenic and urinary chromium concentrations. Values shown are mean  $\pm$  SE. Cut points were determined according to medians (arsenic, 7.7  $\mu\text{g/g}$  creatinine; chromium, 2.0  $\mu\text{g/g}$  creatinine) of urinary creatinine-adjusted levels among all subjects.

# PAH-DNA adducts



← Occup. exposure  
(Low / Intermed. / High)

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