Principi di tossicologia genetica: meccanismi e valutazione

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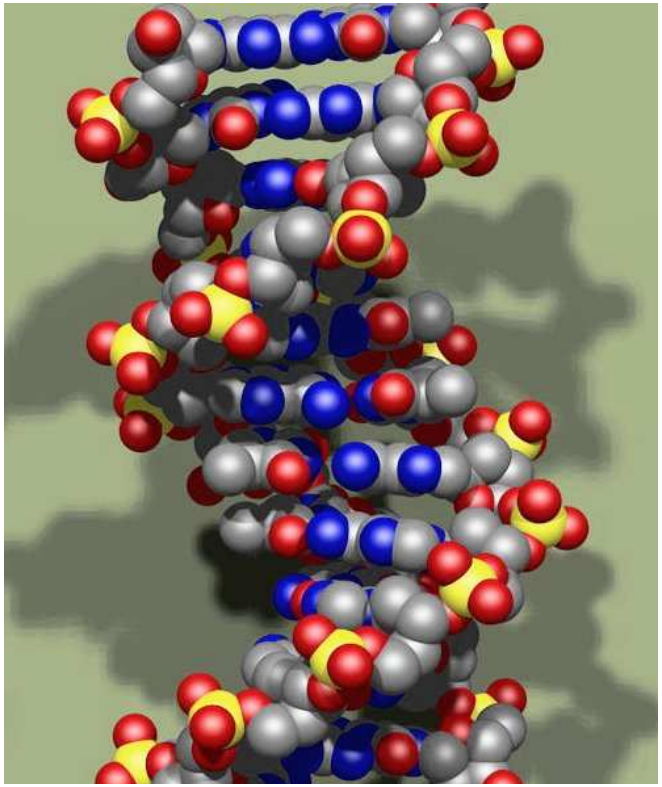
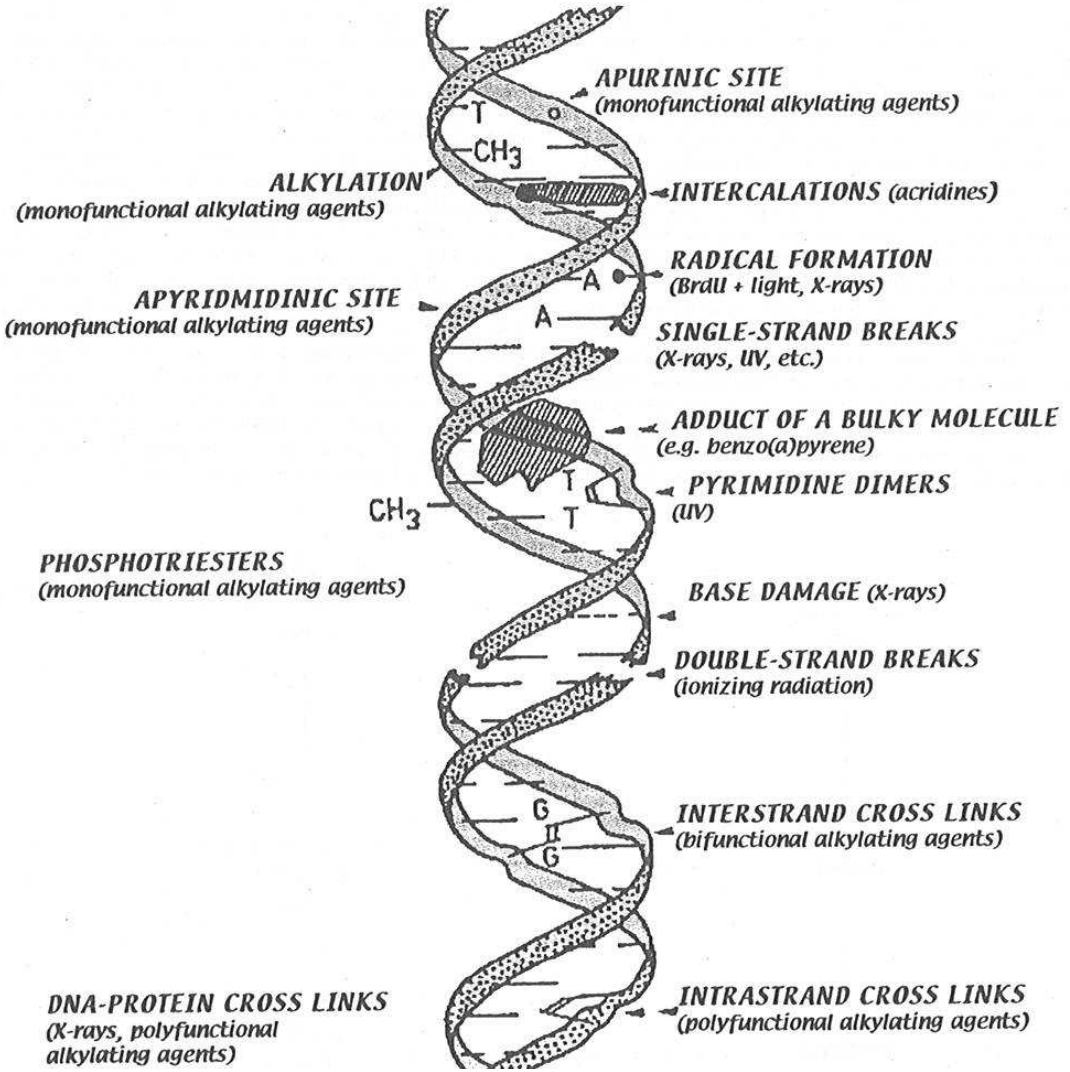
DNA is the informationally active chemical component of all genetic material. The primary structure of DNA is quite dynamic and subject to constant change in the chemistry and sequence of individual nucleotides.

DNA DAMAGE : modifications of the molecular structure of genetic material





SPONTANEOUS ALTERATIONS

MISMATCHES mispairing of bases during DNA synthesis

ALTERATIONS IN THE CHEMISTRY OF DNA BASES

TAUTOMERIC SHIFTS transient rearrangements of bonding

NH_2 (amino) ⟶ NH (imino) (cytosin or adenine)
C=O (keto) ⟶ C-OH (enol) (guanine or thymine)

DEPURINATION

DEAMINATION OF BASES the loss of amino groups occurs spontaneously in pH and temperature reactions of DNA.

cytosine ⟶ uracil
adenine ⟶ hypoxanthine
guanine ⟶ xanthine

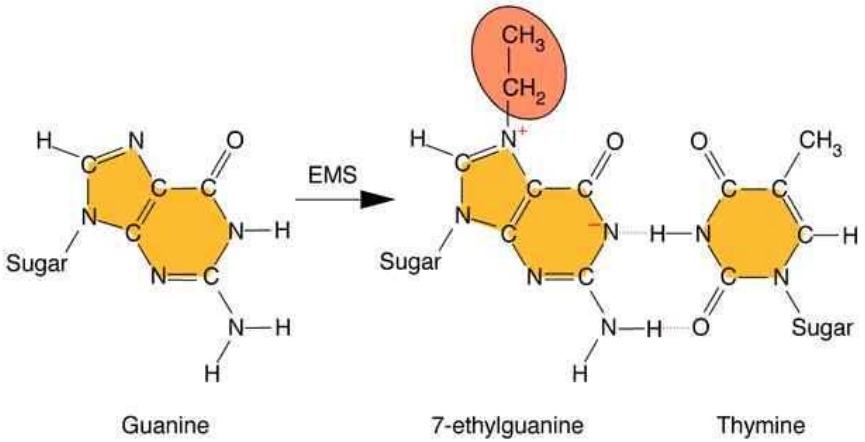
CHEMICAL CARCINOGENS/MUTAGENS

Direct-acting compounds
  reactive electrophiles Es. EMS

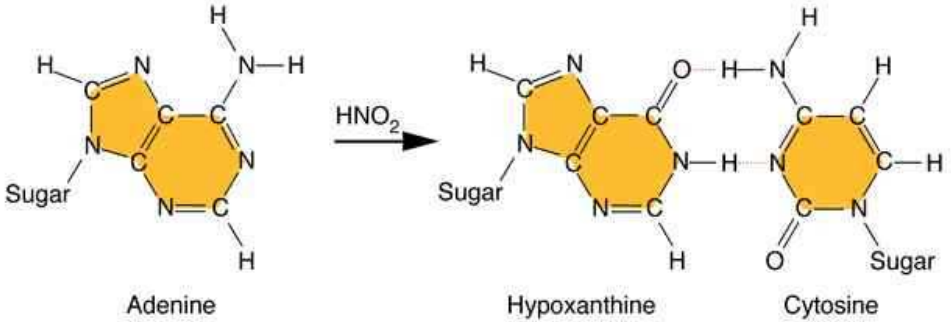
Indirect-acting compounds
  can react with nucleic acid only after conversion to
  reactive electhrophiles by metabolism
  (activation and detoxification)

Mode of action of chemical mutagenic agents

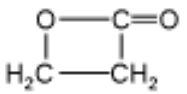
Alkylating agents are chemicals that donate alkyl groups to other molecules. Ethyl methanesulfonate (EMS) is an example.



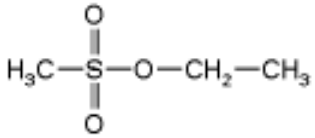
Deaminating agents



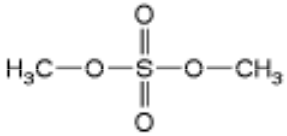
DIRECT-ACTING CARCINOGENS



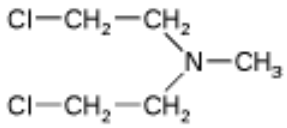
β-Propiolactone



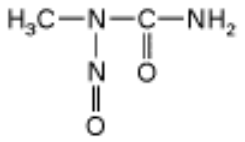
Ethylmethane sulfonate (EMS)



Dimethyl sulfate (DMS)

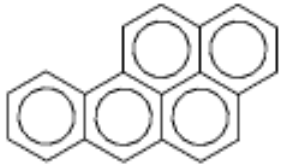


Nitrogen mustard

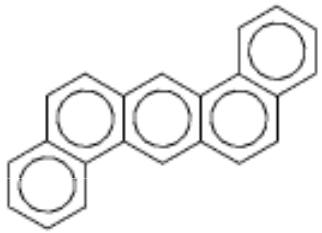


Methyl nitrosourea (MNU)

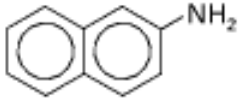
INDIRECT-ACTING CARCINOGENS



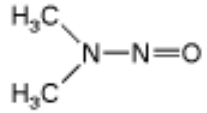
Benzo(*a*)pyrene
(3,4-benzpyrene)



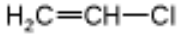
Dibenz(*a,h*)anthracene



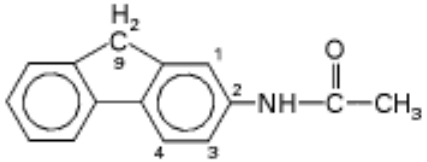
2-Naphthylamine



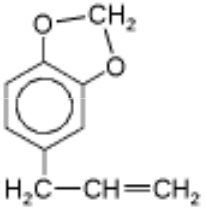
Dimethylnitrosamine



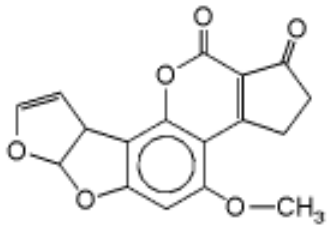
Vinyl chloride



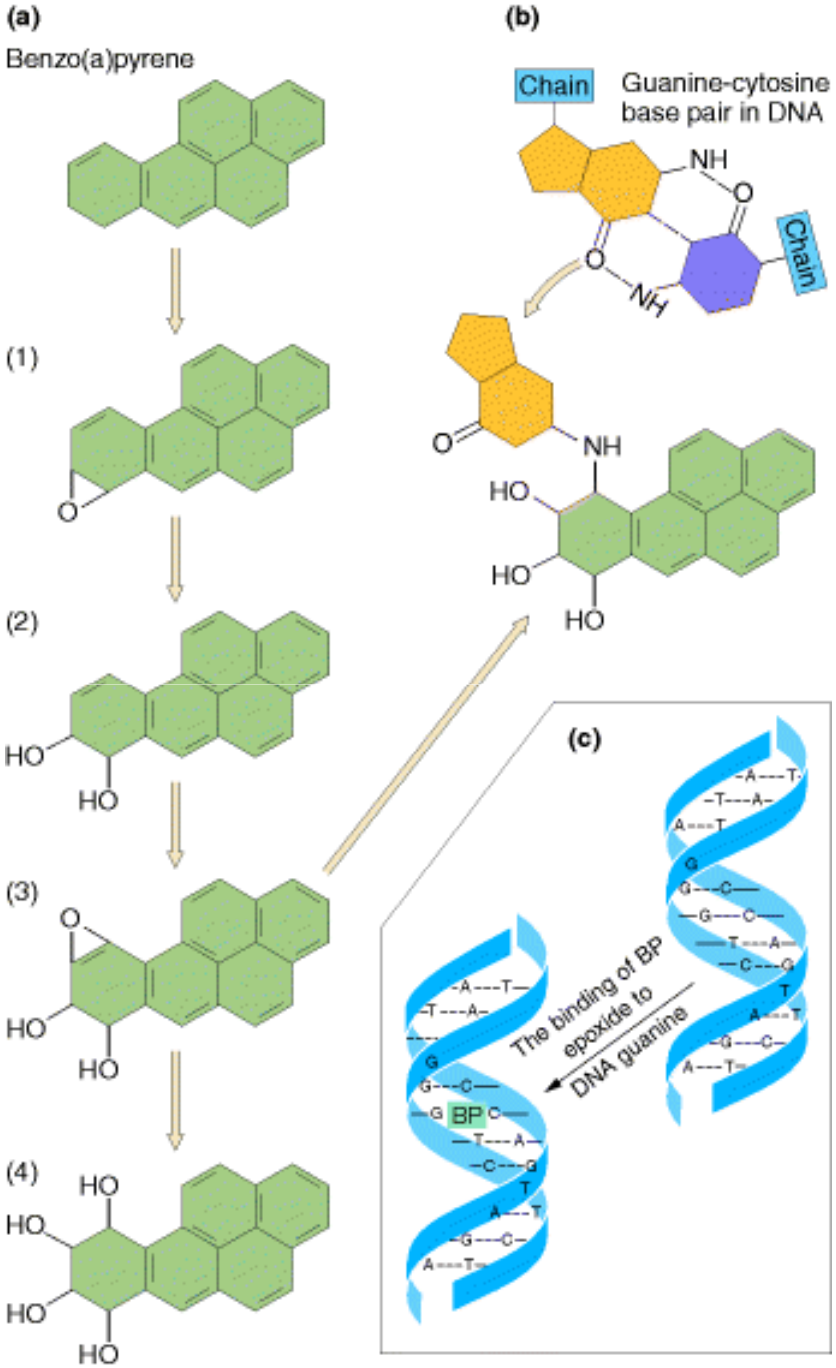
2-Acetylaminofluorene



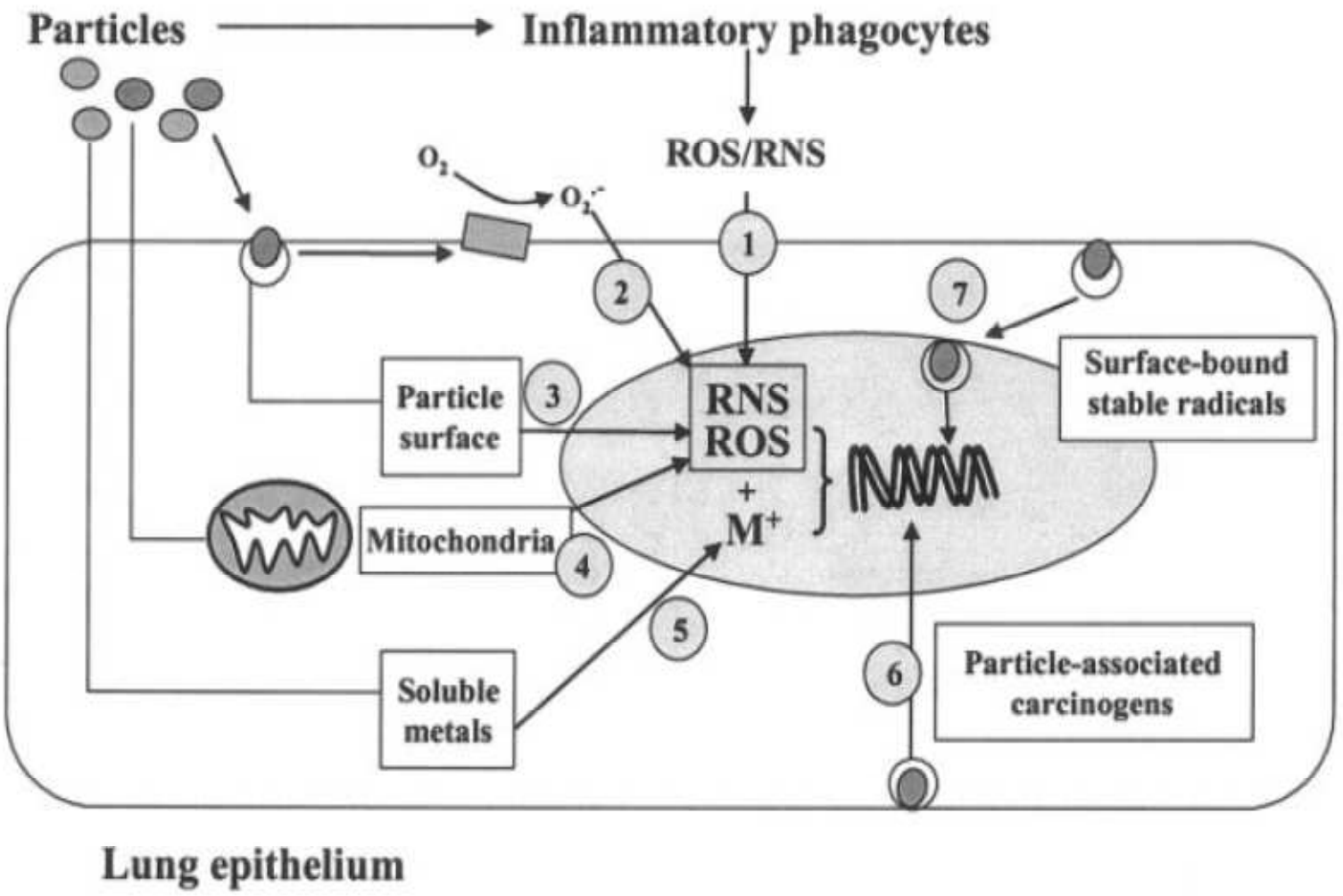
Safrole
(sassafras)



Aflatoxin B_1
(Aspergillus flavus)


(a)
Benzo(a)pyrene
(1)
(2)
(3)
(4)
HO
O
(b)
Chain
Guanine-cytosine
base pair in DNA
NH
Chain
(c)
The binding of BP
epoxide to
DNA guanine
BP

ASBESTOS GENOTOXICITY



Knaapen et , 2004

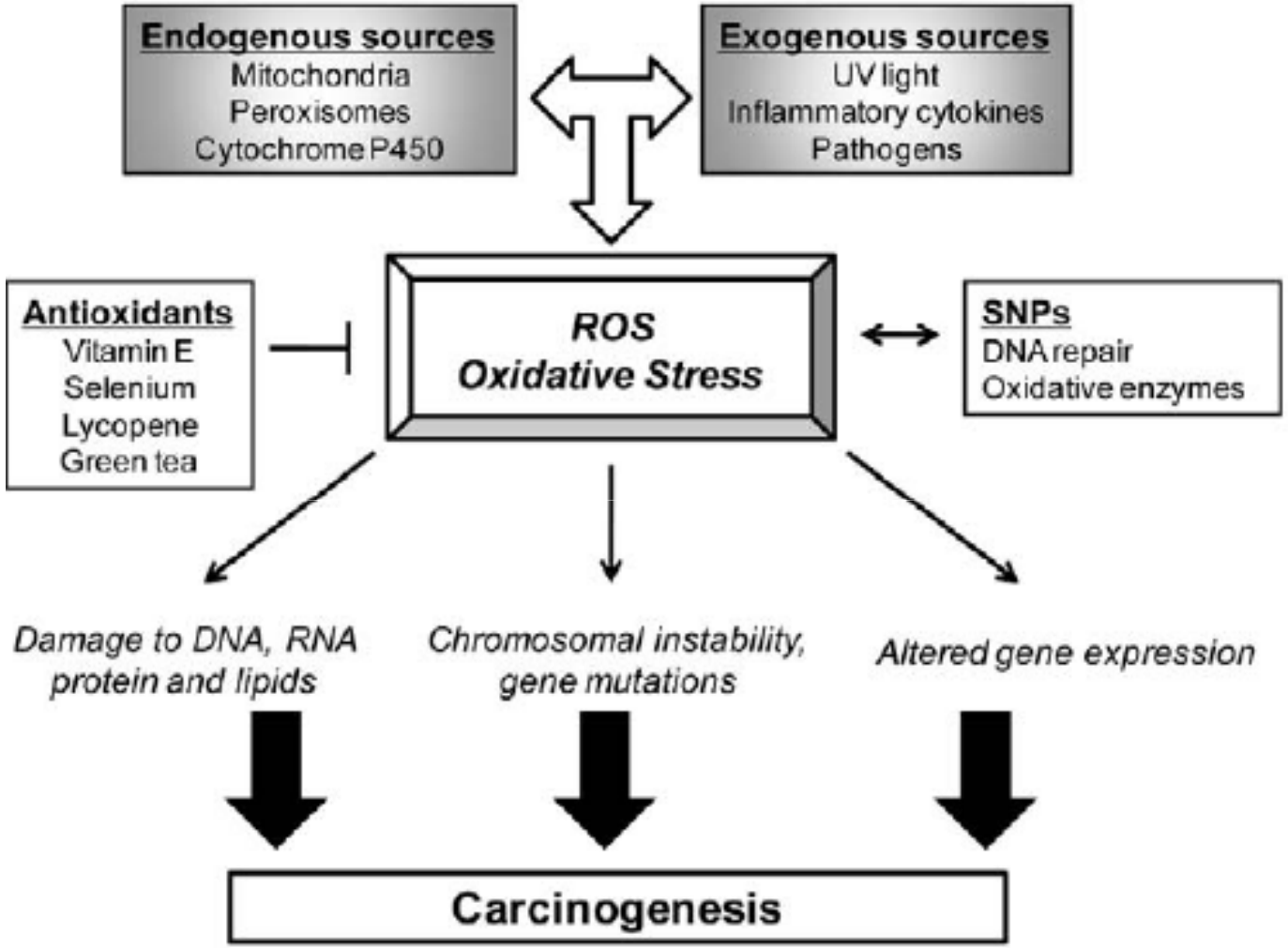
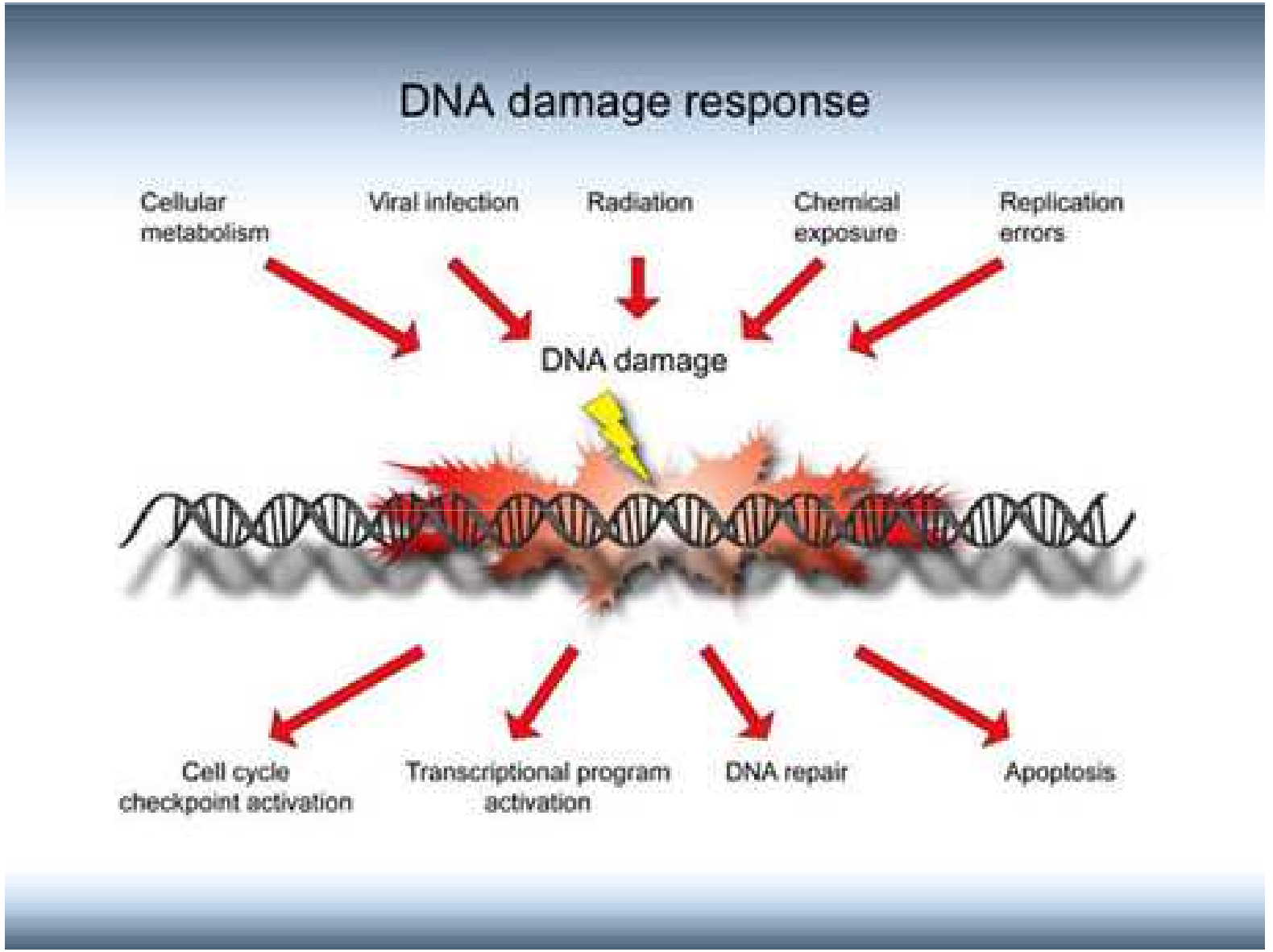
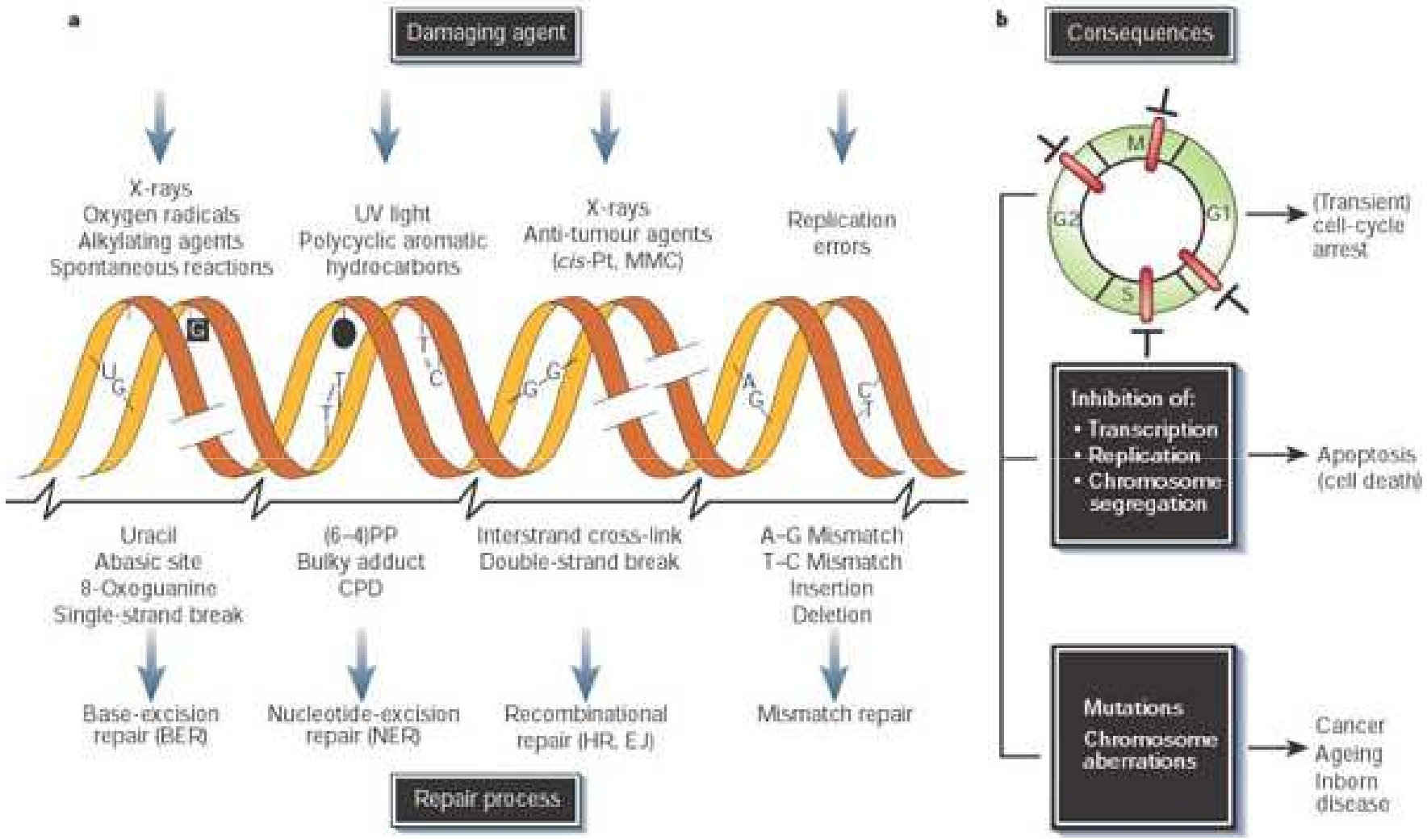

Endogenous sources
Mitochondria
Peroxisomes
Cytochrome P450
Exogenous sources
UV light
Inflammatory cytokines
Pathogens
Antioxidants
Vitamin E
Selenium
Lycopene
Green tea
ROS
Oxidative Stress
SNPs
DNA repair
Oxidative enzymes
Damage to DNA, RNA protein and lipids
Chromosomal instability, gene mutations
Altered gene expression
Carcinogenesis

TABLE 2.—Environmental and pharmaceutical carcinogens that can induce oxidative stress and damage.

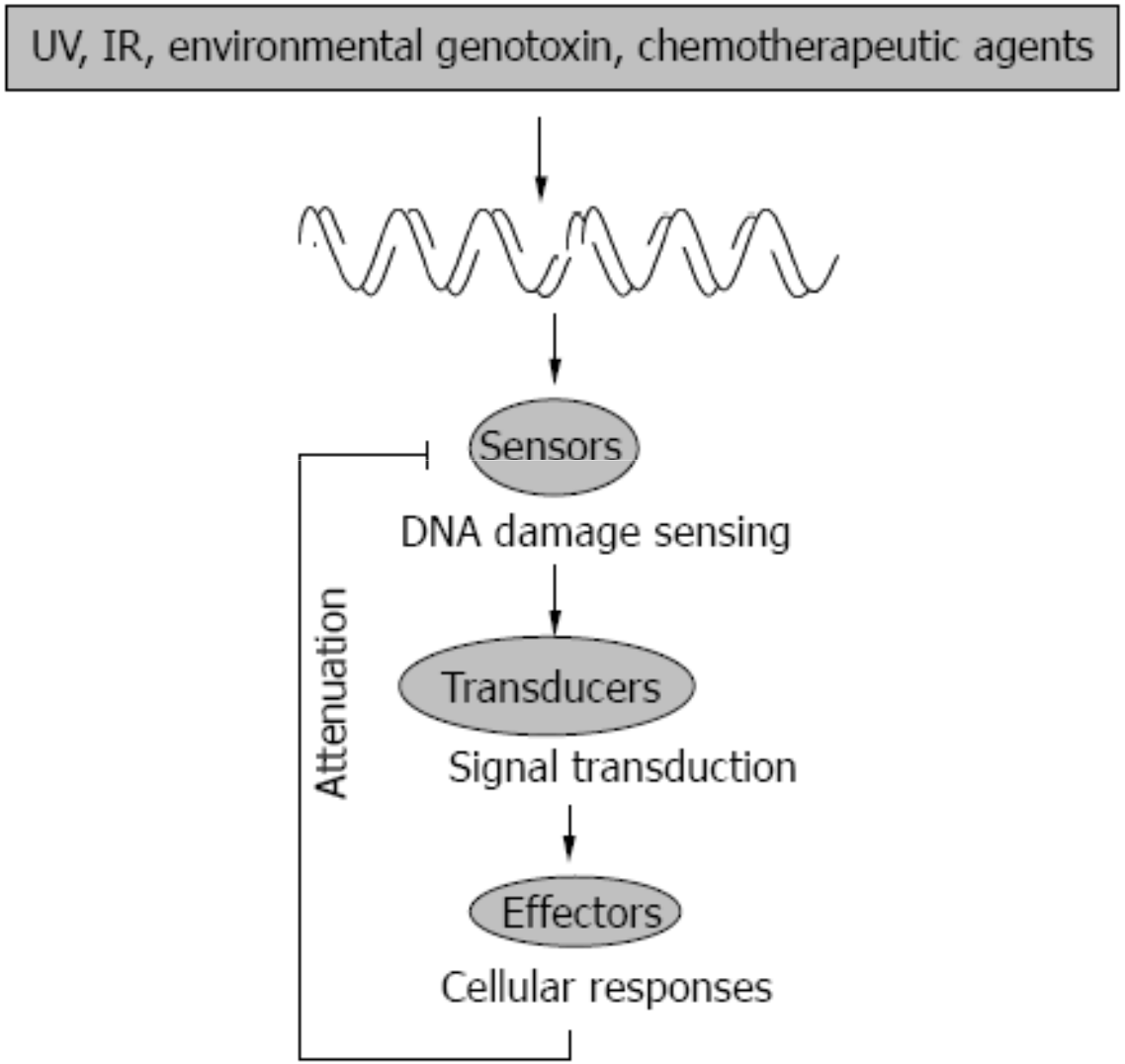
Chemicals	Experimental models	ROS or effects	Reference
Genotoxic			
N-nitroso compounds	Murine	MDA, 8OHdG	Bartsch, Hietanen, and Malaveille (1989); Srinivasan and Glauert (1990); Chung and Xu (1992)
BaP	Mice	8OHdG	Mauthe et al. (1995)
AFB-1	Rats	8OHdG	Shen et al. (1995)
Heterocyclic amines	In vitro	OH	Sato et al. (1992)
MMC and 2-acetylaminofluoene	In vitro	OH	Komiyama, Kikuchi, and Sugiura (1982); Srinivasan and Glauert (1990)
KBrO3	Rats	8OHdG	Umemura et al. (1995)
Nongenotoxic			
2-butoxyethanol	Mice		Siesky, Kamendulis, and Klaunig (2002)
Acrylonitrile	Rats; in vitro	MDA, 8OHdG	Jiang, Xu, and Klaunig (1998); Whysner et al. (1998); Kamendulis et al. (1999)
Chlorinated compounds (TCDD, dieldrin, DTT, lindane)	Murine; in vitro	Lipid peroxidation, $O2^{\cdot -}$, etc.	Videla, Barros, and Junqueira (1990); Junqueira et al. (1991); Alsharif, Lawson, and Stohs (1994)
Phenobarbital	Murine	OH, 8OHdG, lipid peroxidation	Junqueira et al. (1991)
Metal (nickel, BrCl, chromium, Fe-NTA iodobenzene)	Murine	OH, 8OHdG, MDA, NO	Klein, Frenkel, and Costa (1991); Sai et al. (1992); Bagchi and Stohs (1993); Iqbal, Giri, and Athar (1995)
Peroxisome proliferator (DEHP, WY-14,643, clofibrate, ciprofibrate, PFDA)	Murine	OH, 8OHdG, etc.	Srinivasan and Glauert (1990); Tamura et al. (1990); Wada, Marsman, and Popp (1992); Cattley and Glover (1993); C. Y. Huang et al. (1994)
CC14		Trichloromethyl peroxyl radical	Brattin, Glende, and Recknagel et al. (1985)
Phorbol ester (TPA, PMA)	Murine; in vitro	OH, 8OHdG	Witz (1991)
Quinones	V79 cells	8OHdG	Dahlhaus et al. (1995)

Note for ROS = reactive oxygen species.

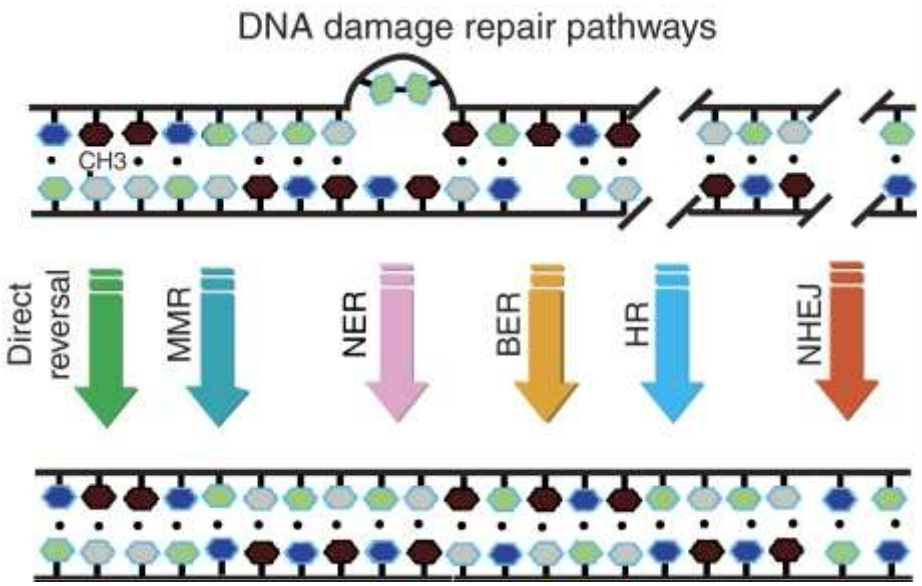

DNA damage response
Cellular metabolism
Viral infection
Radiation
Chemical exposure
Replication errors
DNA damage
Cell cycle checkpoint activation
Transcriptional program activation
DNA repair
Apoptosis


a
Damaging agent
X-rays
Oxygen radicals
Alkylating agents
Spontaneous reactions
UV light
Polycyclic aromatic hydrocarbons
X-rays
Anti-tumour agents (cis-Pt, MMC)
Replication errors
Uracil
Abasic site
8-Oxoguanine
Single-strand break
(6–4)PP
Bulky adduct
CPD
Interstrand cross-link
Double-strand break
A–G Mismatch
T–C Mismatch
Insertion
Deletion
Base-excision repair (BER)
Nucleotide-excision repair (NER)
Recombinational repair (HR, EJ)
Mismatch repair
Repair process
b
Consequences
M
G1
S
G2
(Transient) cell-cycle arrest
Inhibition of:
• Transcription
• Replication
• Chromosome segregation
Apoptosis (cell death)
Mutations
Chromosome aberrations
Cancer
Ageing
Inborn disease

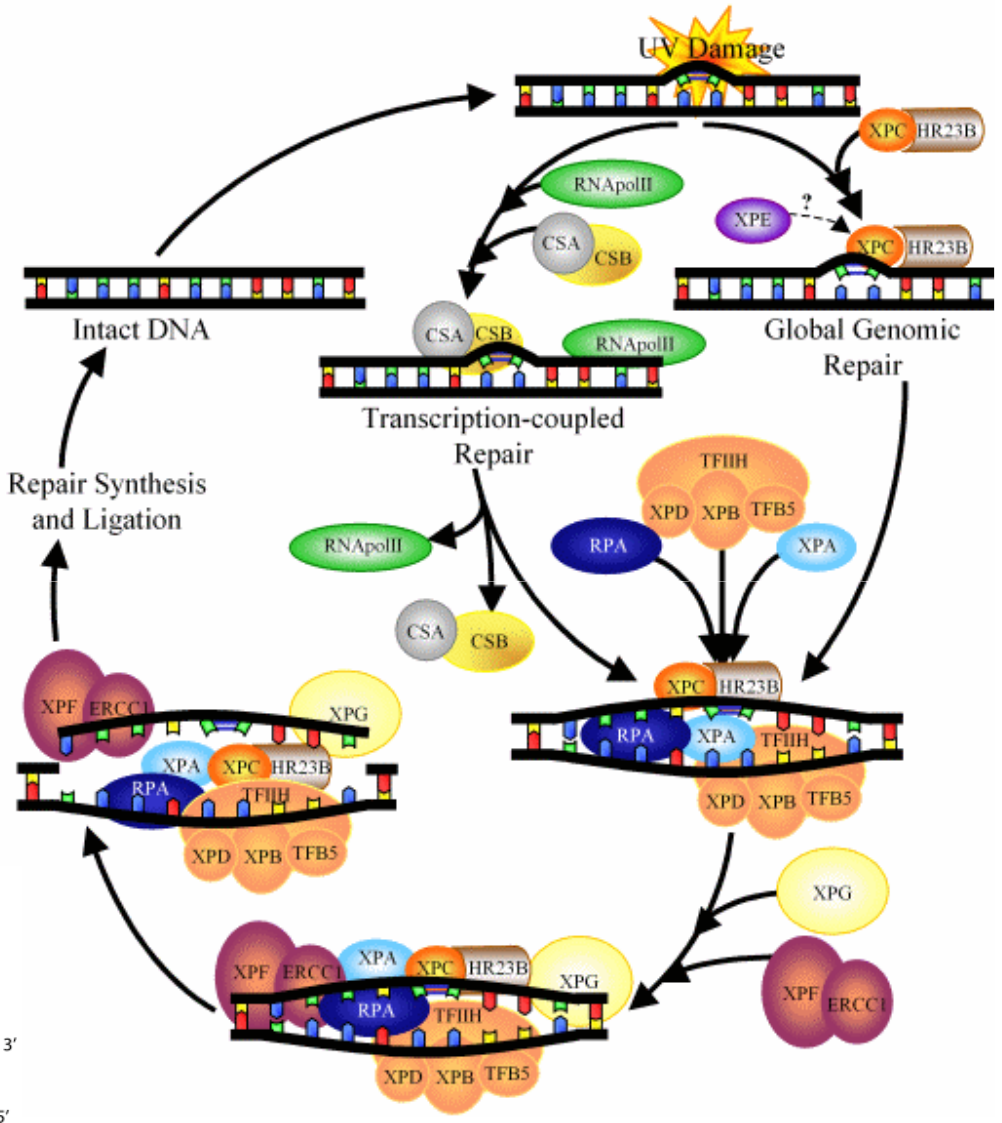
Cellular response to genotoxic damage



DNA Repair

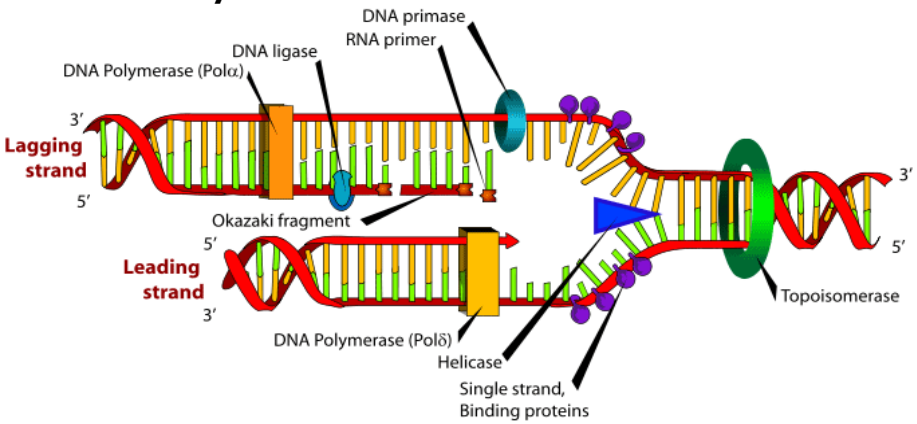


NER

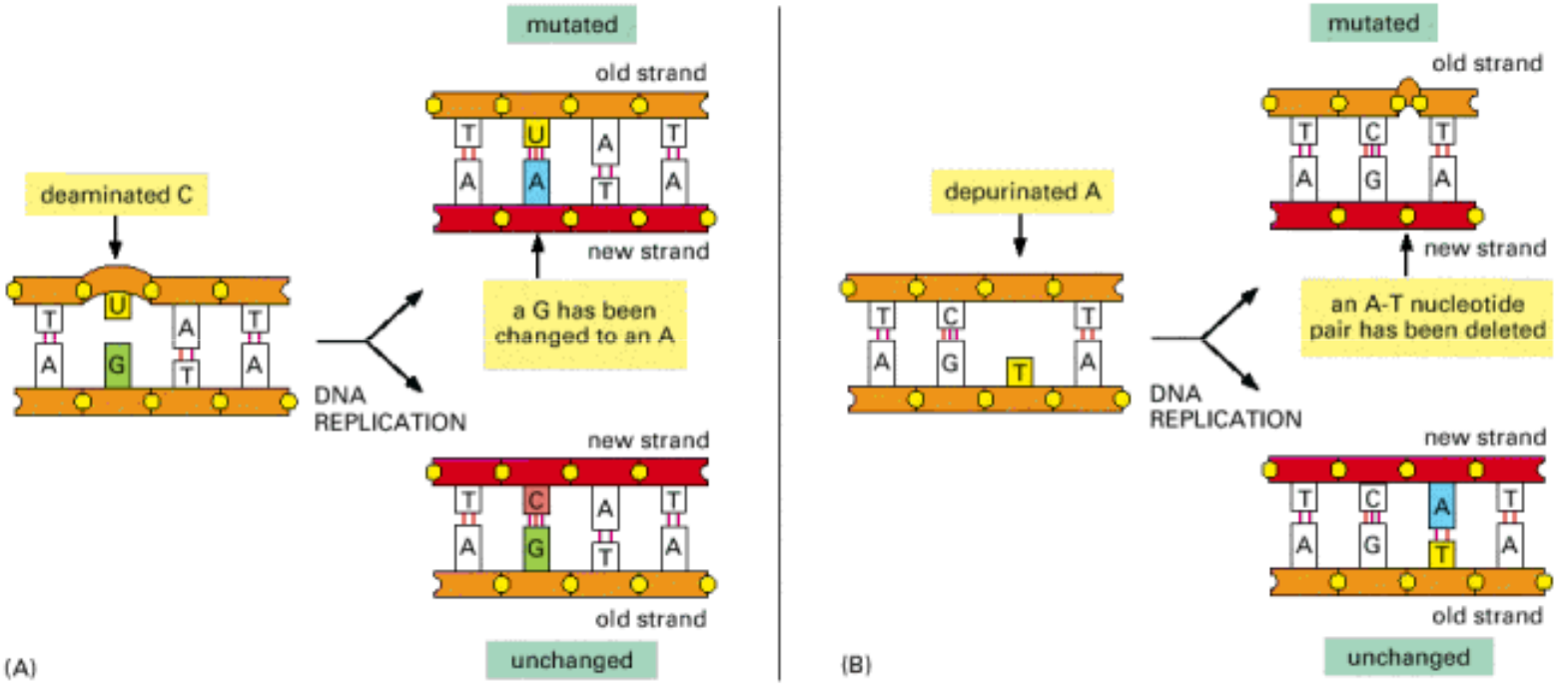


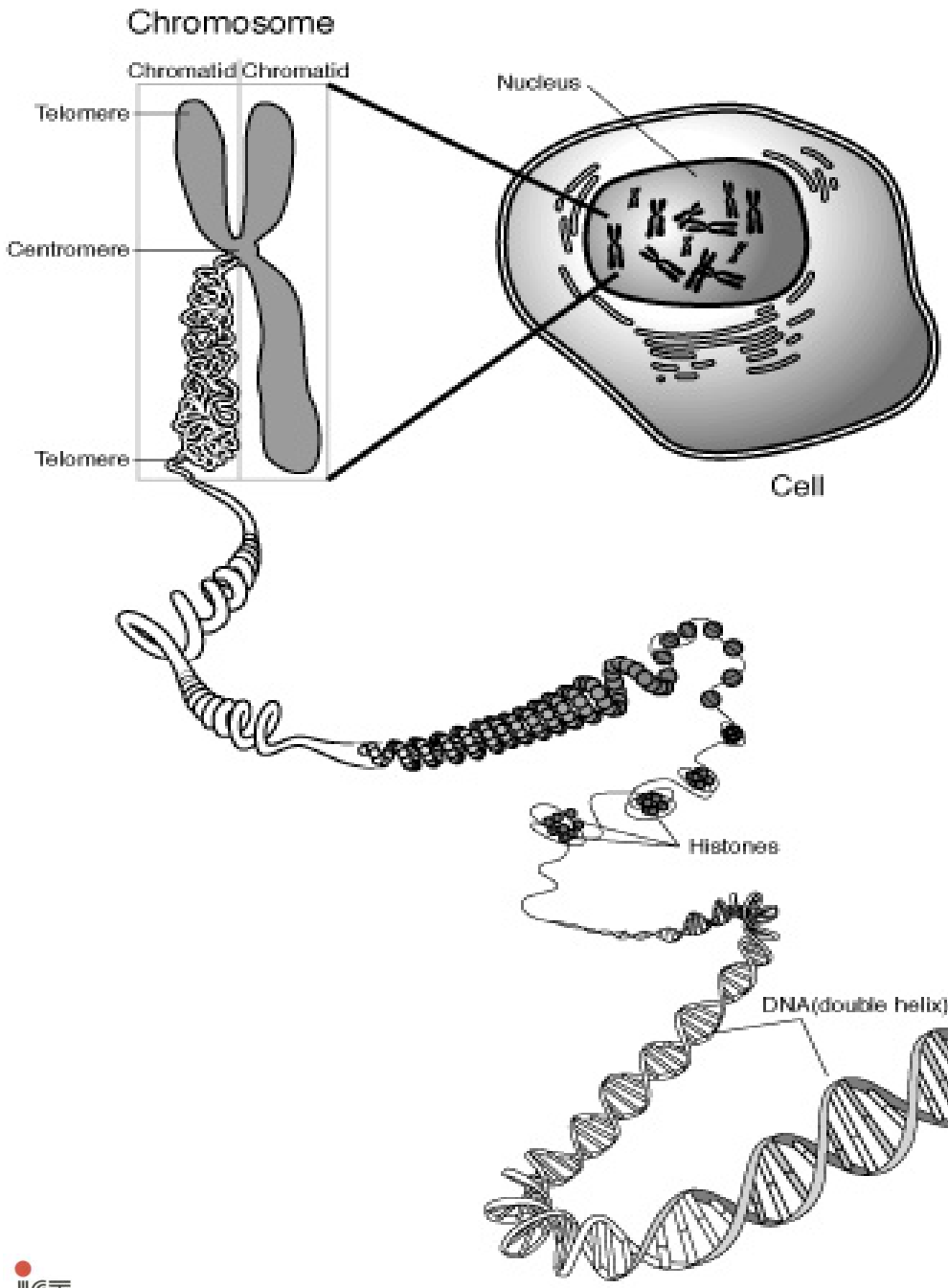
DNA synthesis

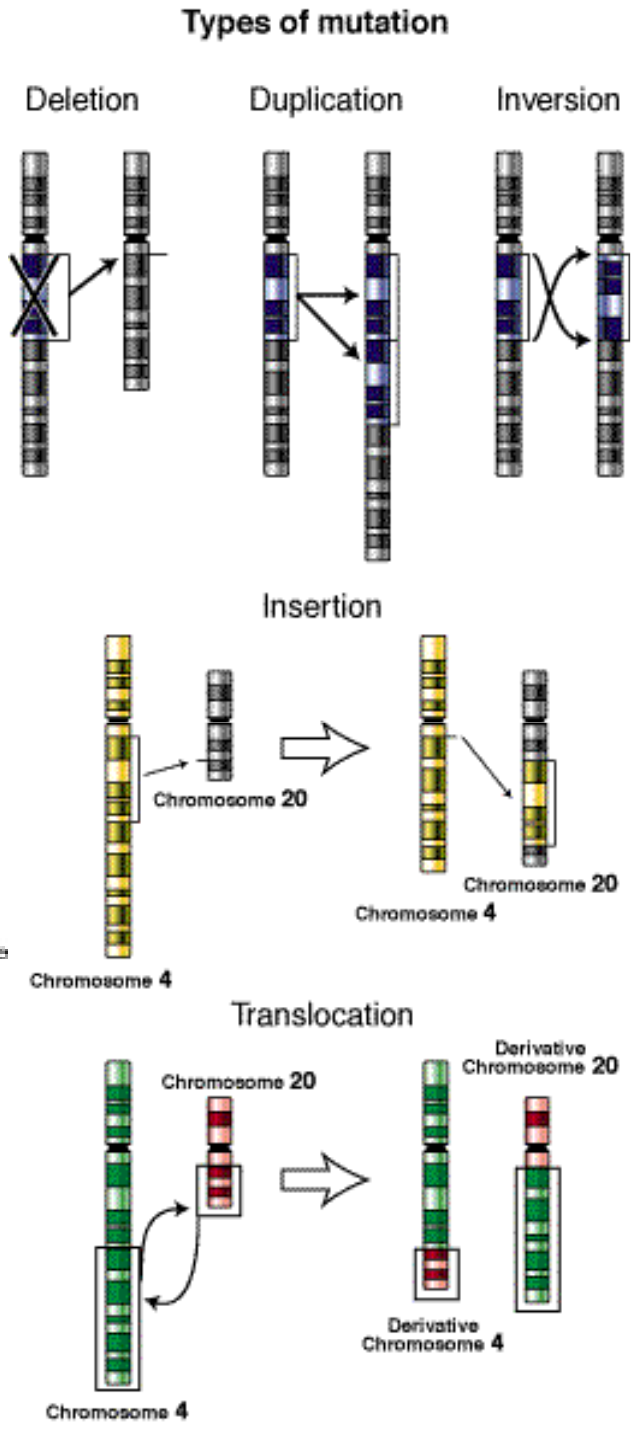
During cell division, the DNA in a cell makes a copy or replica of itself. During this complex process, mistakes may occur.



MUTATIONS


mutated
old strand
deaminated C
new strand
a G has been changed to an A
DNA REPLICATION
new strand
old strand
unchanged
(A)
mutated
old strand
depurinated A
new strand
an A-T nucleotide pair has been deleted
DNA REPLICATION
new strand
old strand
unchanged
(B)


Chromosome
Chromatid
Chromatid
Telomere
Centromere
Telomere
Nucleus
Cell
Histones
Base
DNA(double helix)


Types of mutation
Deletion
Duplication
Inversion
Insertion
Chromosome 20
Chromosome 4
Chromosome 20
Chromosome 4
Translocation
Chromosome 20
Derivative
Chromosome 20
Derivative
Chromosome 4
Chromosome 4

Mutagen an agent that lead to an increase in the frequency of occurrence of mutations

Mutant frequency proportion of mutants in a population

Mutation rate mutations giving a particular scorable phenotype per DNA replication

Genomic mutation mutations per genome per DNA replication.

Point mutations mutations that result from the substitution of one base pair for another or from the addition or deletion of a small number of base pair

Transition or purine or one pyrimidine for another

Transversion interchange of a purine for a pyrimidine or interchange of a pyrimidine for a purine

Frameshift mutations addition or deletion of small number of base pairs that cause a sfift in the translational reading frame.

Mutations and cancer

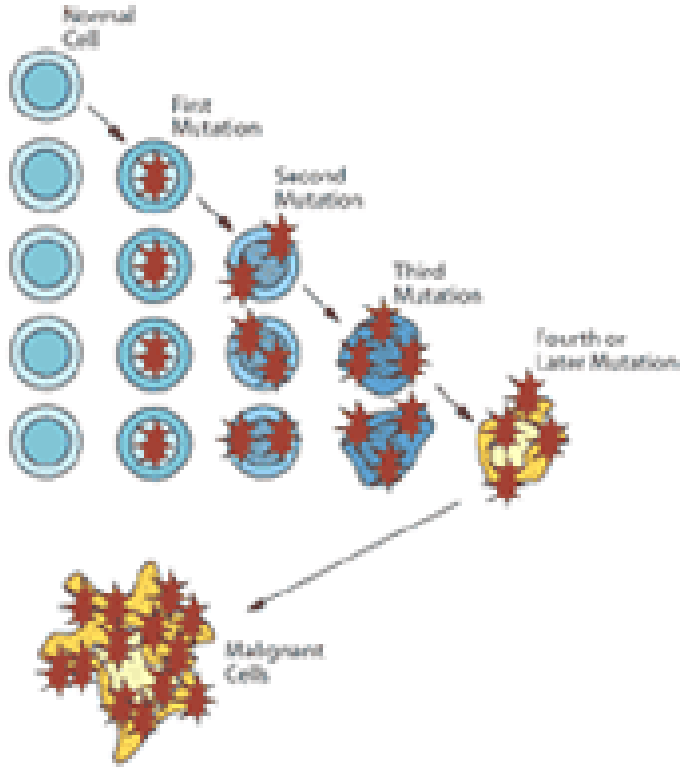
Tumors are characterized by mutations.
Human tumorigenesis proceeds by the accumulation of inherited and somatic mutations:
• gene mutations: insertions, deletions, recombinations
• chromosomal mutations: dicentrics, rings, translocations, double minutes chromosomes, aneuploidy)

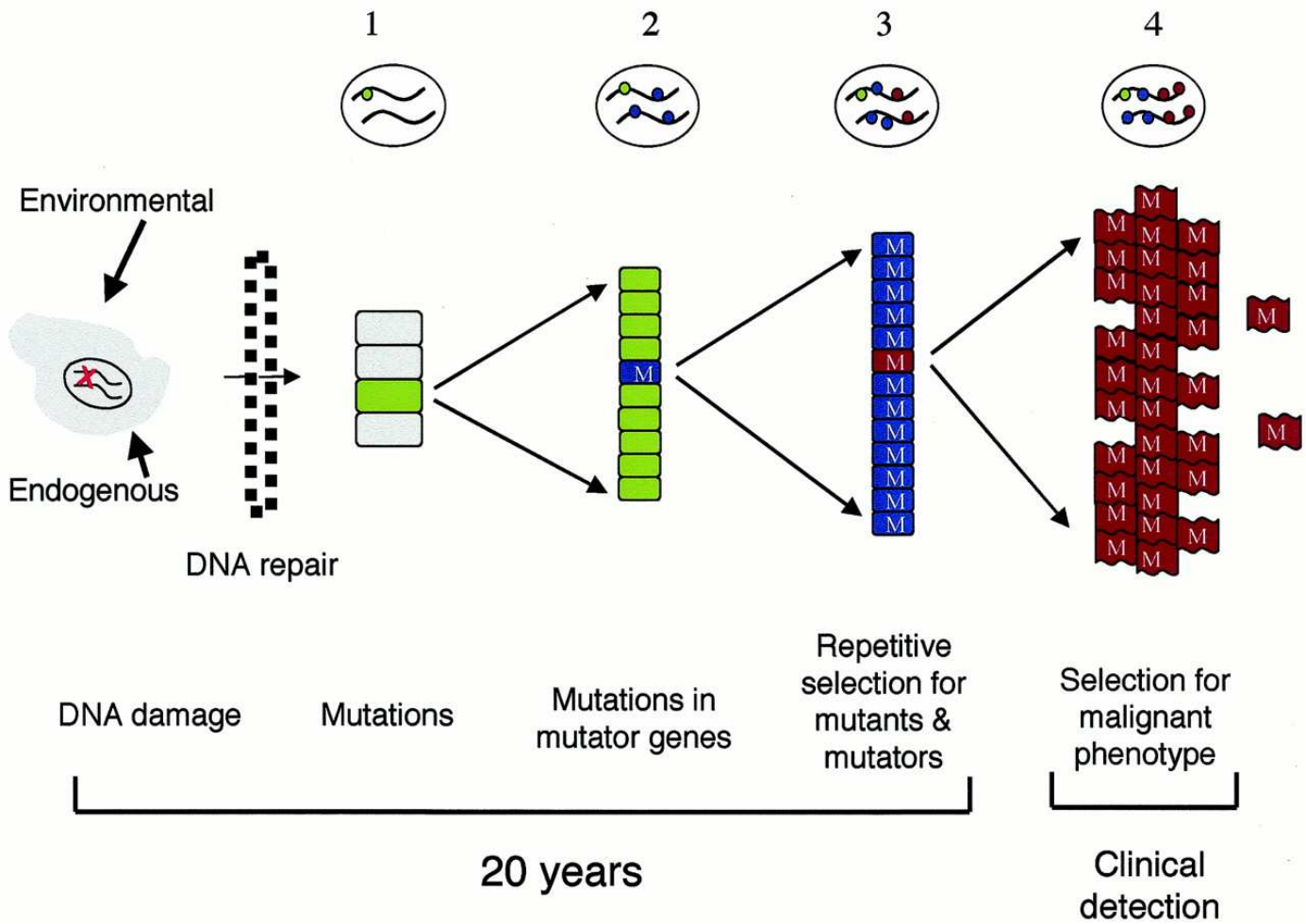
Boveri (1902) showed that abnormal development in sea urchin embryogenesis is associated with chromosomal alterations. He surmised that these changes are analogous to the multiple defects in intercellular cooperation seen in cancers.

Mutation and Cancer

Mutations in key regulatory genes (tumor suppressors and proto-oncogenes) alter the behavior of cells and can potentially lead to the unregulated growth seen in cancer.

For almost all types of cancer studied to date, it seems as if the transition from a normal, healthy cell to a cancer cell is step-wise progression that requires genetic changes in several different oncogenes and tumor suppressors. For almost all types of cancer studied to date, it seems as if the transition from a normal, healthy cell to a cancer cell is step-wise progression that requires genetic changes in several different oncogenes and tumor suppressors.





Loeb, L. A. Cancer Res 2001;61:3230-3239

Cancer Research

Proto-oncogene activation

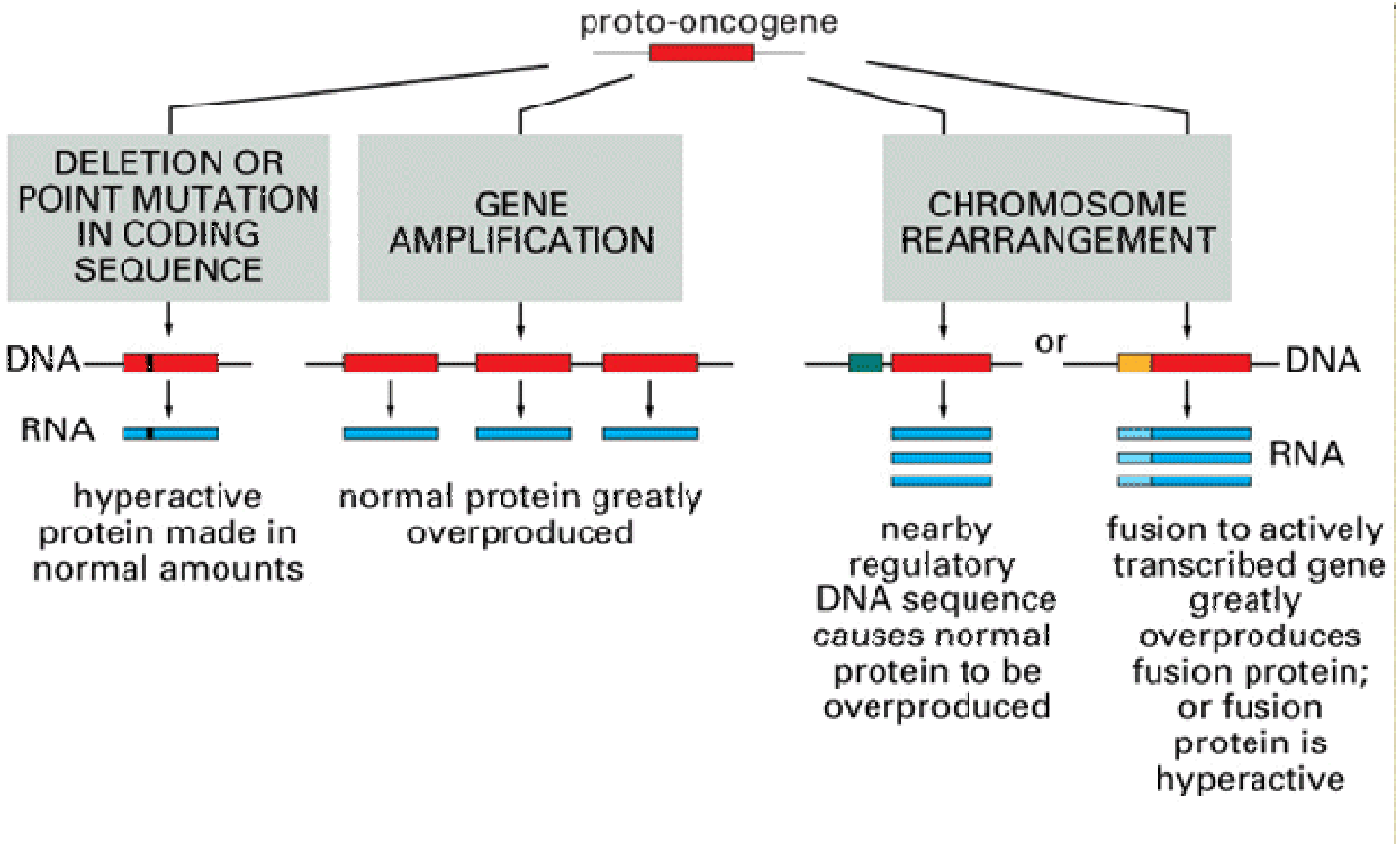
Oncogenes

These are altered forms of genes known as proto-oncogenes. Proto-oncogenes are responsible for promoting cell growth. When altered or mutated, they become oncogenes and then can promote tumor formation or growth. Mutations in proto-oncogenes are usually acquired.

Proto-oncogenes are often involved in signal transduction and execution of mitogenic signals, usually through their protein products.

Having a mutation in just one of the two copies of a particular proto-oncogene is enough to cause a change in cell growth and the formation of a tumor. For this reason, oncogenes are said to be "dominant" at the cellular level.

Examples of proto-oncogenes include: RAS, MYC, WNT, ERK; TKR



Tumour suppressor genes

- The gene's normal function is to regulate cell division. Both alleles need to be mutated or removed in order to lose the gene activity.

- The first mutation may be inherited or somatic.

- The second mutation will often be a gross event leading to loss of heterozygosity in the surrounding area.

p53

- suppresses progression through the cell cycle in response to DNA damage

- initiates apoptosis if the damage to the cell is severe

- is a transcription factor and once activated, it represses transcription of one set of genes (several of which are involved in stimulating cell growth) while stimulating expression of other genes involved in cell cycle control

Role of genotoxicity tests in risk assessment

- **Identify compounds with potential heritable effects**
- **Predict carcinogenic potential**
- **Provide informations on mode of action of carcinogenic agents**

Genotoxic (or genotoxicity) refers to agents which interact with the DNA and/or the cellular apparatus which regulates the fidelity of the genome, eg the spindle apparatus, and enzymes such as the topoisomerases.

It is a broad term that includes mutation as well as damage to DNA or the production of DNA adducts, by the chemical itself or its metabolites.

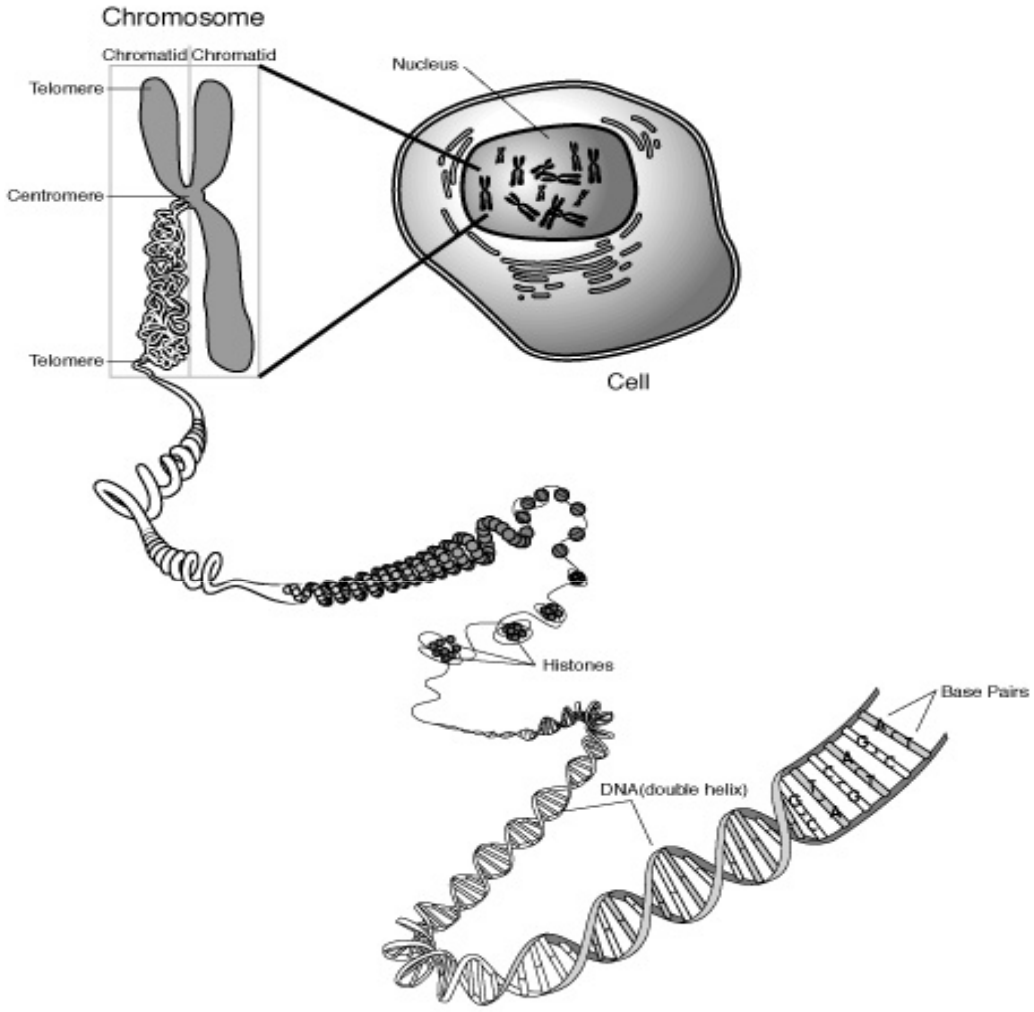
Mutation refers to a permanent change in the amount or structure of the genetic material of an organism, which may result in a heritable change in the characteristics of the organism. These alterations may involve:individual genes, blocks of genes, or whole chromosomes.

Gene mutation

Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of larger changes, including deletions and rearrangements of DNA.

Chromosomal mutation

Changes involving chromosomes as entities may be numerical or structural.

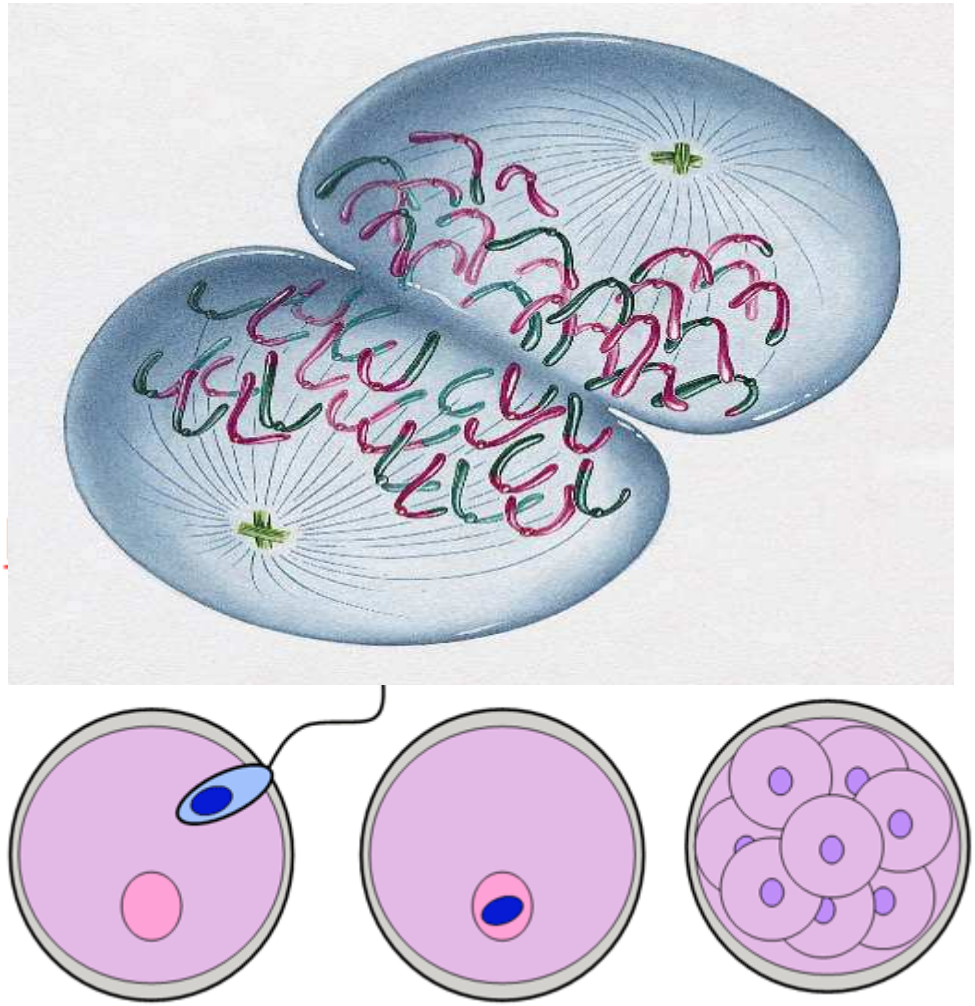


Clastogenicity **is used for agents giving rise to structural chromosome aberrations. A clastogen can cause breaks in chromosomes that result in the loss or rearrangements of chromosome segments.**

Aneugenicity **(aneuploidy induction) refers to the effects of agents that give rise to a change (gain or loss) in chromosome number in cells. An aneugen can cause loss or gain of chromosomes resulting in cells that have not an exact multiple of the haploid number**

Germ cell mutation

A mutation in the germ cells of sexually reproducing organisms may be transmitted to the offspring, whereas a mutation that occurs in somatic cells may be transferred only to descendent daughter cells. Mutagenic chemicals may present a hazard to health since exposure to a mutagen carries the risk of inducing germ-line mutations, with the possibility of inherited disorders, and the risk of somatic mutations including those leading to cancer.



GENE MUTATION TESTS

- ✓**Salmonella reverse mutation (Ames Test)**
  E.coli reverse mutation

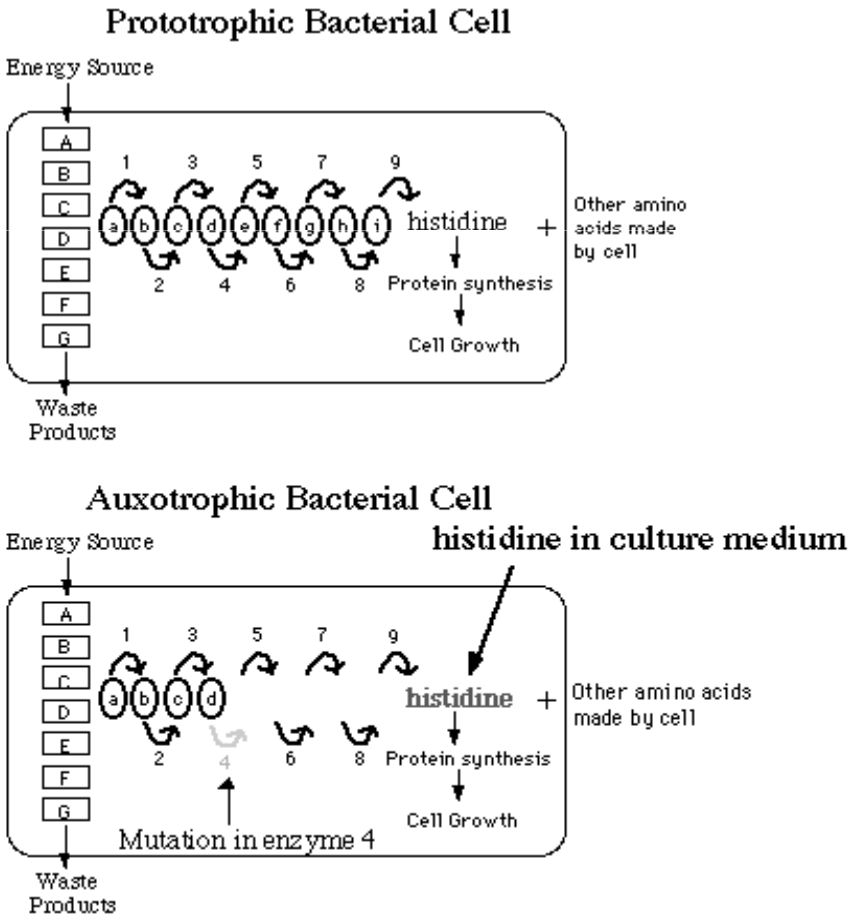
- ✓**Mammalian Cell Mutation Assay**
  forward mutations HPRT, ouabain resistance etc

- ✓**Gene mutations in vivo in transgenic mice**

AMES TEST OECD 471 (1997)

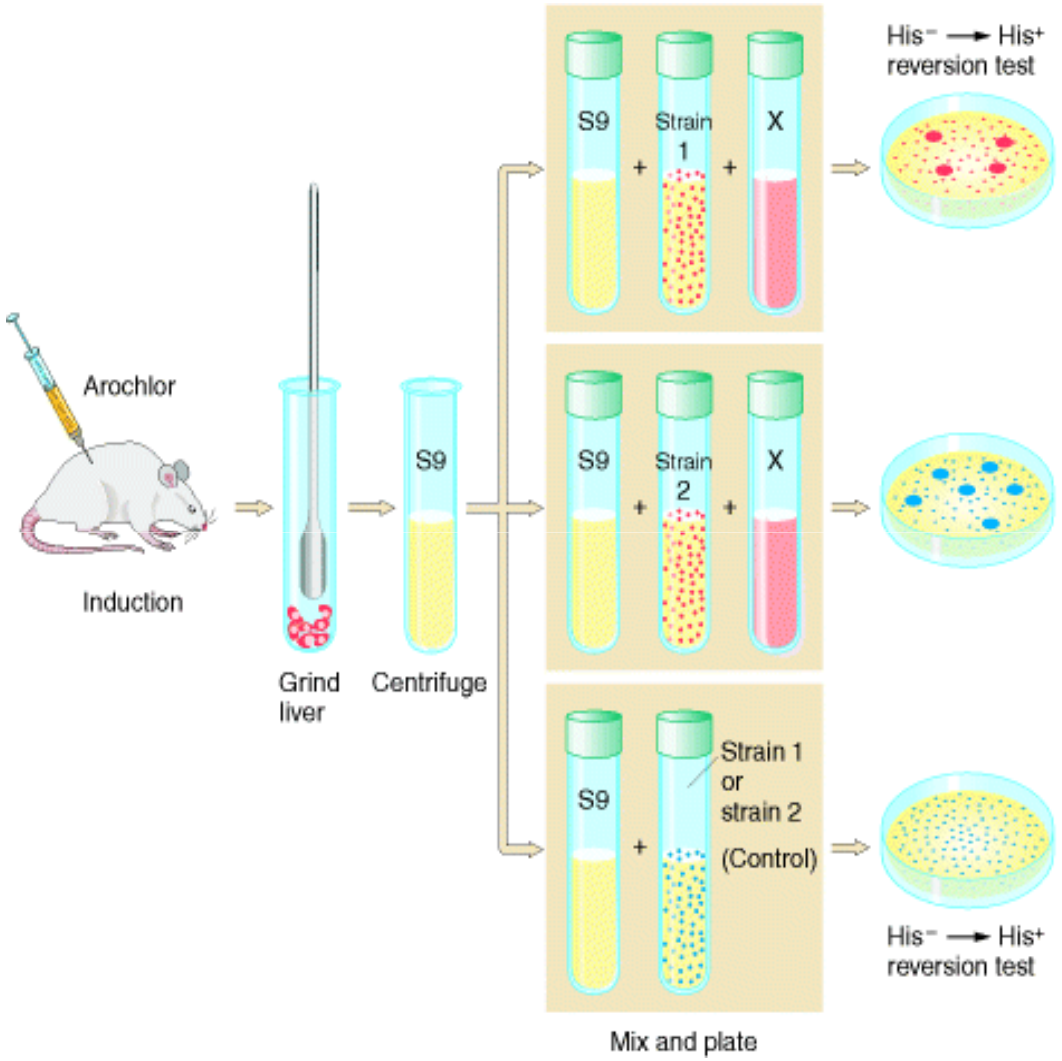
The Ames test uses a mutant strain of *Salmonella typhimurium* which cannot grow in the absence of the amino acid histidine because a mutation has occurred in a gene that encodes one of the nine enzymes used in the pathway of histidine synthesis.

These auxotrophic mutants are called *histidine-dependent* or his -mutants because they depend on an external source of histidine to grow.
Auxotrophs are mutant individuals that cannot make all the metabolic products that wild-type (prototrophic) individuals



AMES TEST

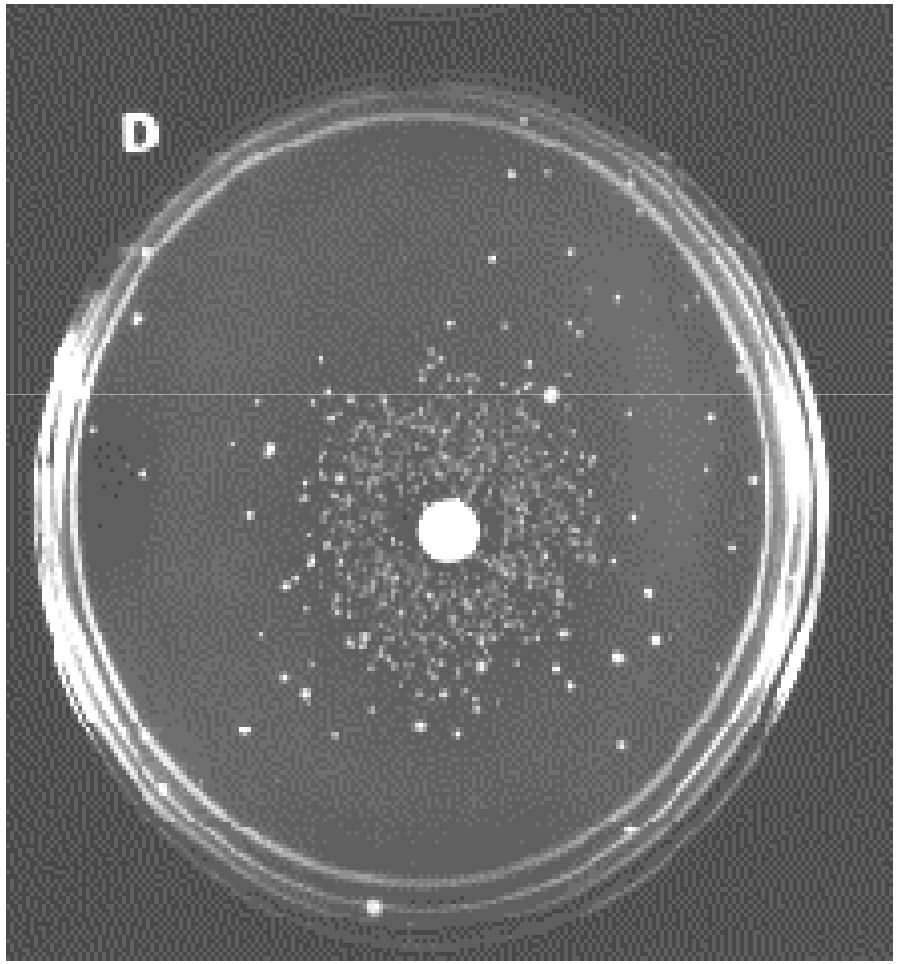
The bacterium used in the test is a strain of Salmonella typhimurium that carries a defective (mutant) gene making it unable to synthesize amino acid histidine (His) from the ingredients in its culture medium. However, some types of mutations (including this one) can be reversed, a back mutation, with the gene regaining its function. These revertants are able to grow on a medium lacking histidine.



Ames Test

Developed by Bruce Ames and his colleagues in the 1970s.

- The picture (courtesy of Bruce Ames) shows a qualitative version of the Ames test. A suspension of a histidine-requiring (His−) strain of Salmonella typhimurium has been plated with a mixture of rat liver enzymes on agar lacking histidine. The disk of filter paper has been impregnated with 10µg of 2-aminofluorene, a known carcinogen. The mutagenic effect of the chemical has caused many bacteria to regain the ability to grow without histidine, forming the colonies seen around the disk. The scattered colonies near the margin of the disk represent spontaneous revertants.



***S. Typhimurium* strain**	**Gene Affected**	**DNA-repair**	**LPS**	**Biotin Requirement**	**Plasmide**	**Mutation Event**
S. Typh. TA100	hisG	uvrB	rfa	bio-	pkM101	base-pair substitution
S. Typh. TA102	hisG	-	rfa	bio-	pkM101 pAQ1	base-pair substitution
S. Typh. TA1535	hisG	uvrB	rfa	bio-	-	base-pair substitution missense
E. Coli WP2 uvrA	trp	uvrA	-	-	-	base-pair substitution
S. Typh. TA98	hisD	uvrB	rfa	bio-	pkM101	frameshift insertion
S. Typh. TA1537	hisC	uvrB	rfa	bio-	-	frameshift deletion
S. Typh. TA1538	hisC	uvrB	rfb	bio-	-	frameshift insertion

In vitro assay for gene mutations in mammalian cells

- **Culture set up**
- **Cytotoxicity test**
- **Treatment with the test compound in the presence and absence of metabolic activation system**
- **Expression period during which mutations are fixed in DNA and the endogenous levels of wild enzyme decrease**
- **Selection period during which cells are cloned in the presence of a selective agent**

MAMMALIAN CELLS: FORWARD MUTATION ASSAYS
OECD 476

- **Hprt locus hypoxantinephosphoribosyl transferase**
  resistance to 6-thioguanine
  CHO chinese hamster ovary cells
  V79 chinese hamster lung cells

- **Na/K- ATPase**
  resistance to ouabain
  CHO chinese hamster ovary cells
  V79 chinese hamster lung cells

- **tk locus-thymidine kinase**
  resistance to threefluorotymidine
  L5178Y mouse lymphoma cells

CHO-HGPRT mutation assay

HGPRT enzyme catalyzes phosphorylation of purines in one of the purine salvage pathway.
6-TG is a substrate for this enzyme.
Cells retaining the enzyme are susceptible to the cytoxic effects of 6-TG

Forward mutations that result in a loss of the functional HPRT gene render the cells resistant to 6-TG.

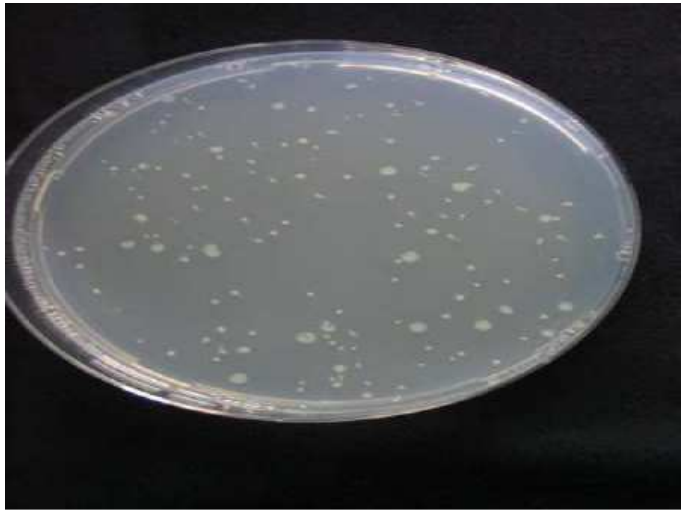
The mutant cells can be quantitated by cloning the cells in culture medium supplemented with 6-TG, the selective agents.

TK mouse lymphoma mutation assay

Utilizes a strain of mouse lymphoma cells that has made heterozygous at the TK locus.

TK enzyme catalyzes phosphorylation of thymidine in one of the salvage pathway.
Trifluorothymidine (TFT) is the selective agent: it can be phosphorylated by the TK enzyme and cells containing the enzyme are susceptible to the cytoxic effects of TFT.

Forward mutations that result in a loss of TK enzyme and acquisition of TFT resistance.



Gene mutations

Transgenic mice **BIG BLUE MICE**

Environmental Health Criteria 233

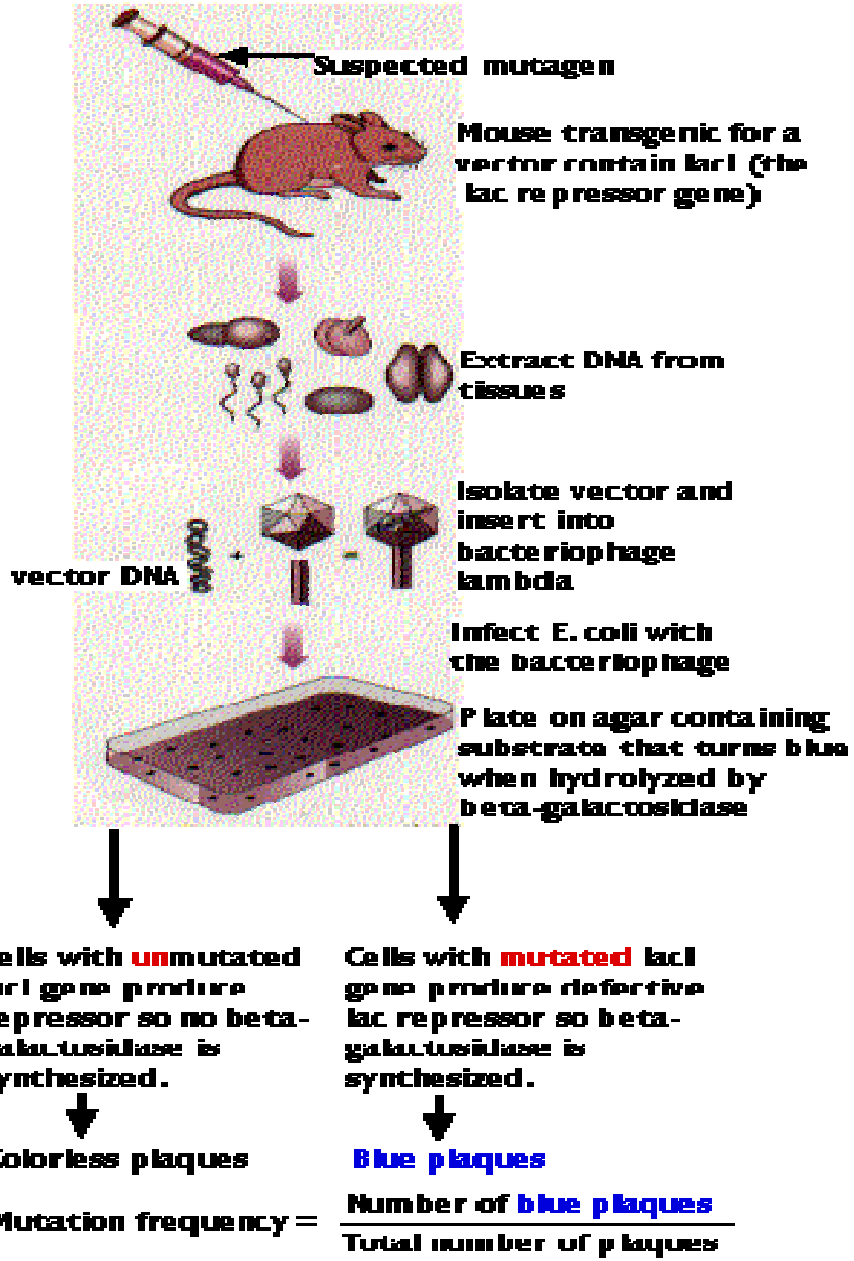
A transgenic animal carries a foreign gene that has been deliberately inserted into its genome.

The foreign gene is constructed using recombinant DNA methodology. In addition to a **structural gene**, the DNA usually includes other sequences to enable it to be incorporated into the DNA of the host and to be expressed correctly by the cells of the host.

Big Blue mice are transgenic for a segment of DNA that contains:

the DNA of **bacteriophage lambda**, a virus that infects E. coli, and which serves here as the vector for 3 genetic elements from the **lac operon** of E. coli:

- The lacI gene
- the **operator** of the operon
- the beta galactosidase (lacZ)



Chromosomal Aberration tests

Mammalian Chromosome Aberration Test In Vitro
OECD TG 473*(1997)*

Mammalian Bone Marrow Chromosome Aberration Test
OECD 475 (1997)

Mammalian Erythrocyte Micronucleus Test
OECD 474 (1997)

In vitro micronucleus test
OECD 487 (2004)

Mammalian Chromosome Aberration Test In Vitro OECD TG 473*(1997).*

AIMS:
Structural chromosome damage: Chromatid-type aberrations
Chromosome-type aberrations

Numerical aberrations: change in the number of chromosomes

Method:
Cells
A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

Exposure
Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system.

Doses: defined by a previous cytotoxicity test. At least three analysable concentrations should be use
Concurrent positive and negative (solvent or vehicle) controls.
the maximum test concentration should be 5 µl/ml, 5 mg/ml or 0.01 M

Mammalian Bone Marrow Chromosome Aberration Test OECD 475 (1997)

Aims

The mammalian in vivo chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of rodents (mice or rats)

Method

Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment.
Prior to sacrifice, animals are treated with a metaphase-arresting agent (e.g., colchicine or Colcemid®).

Doses

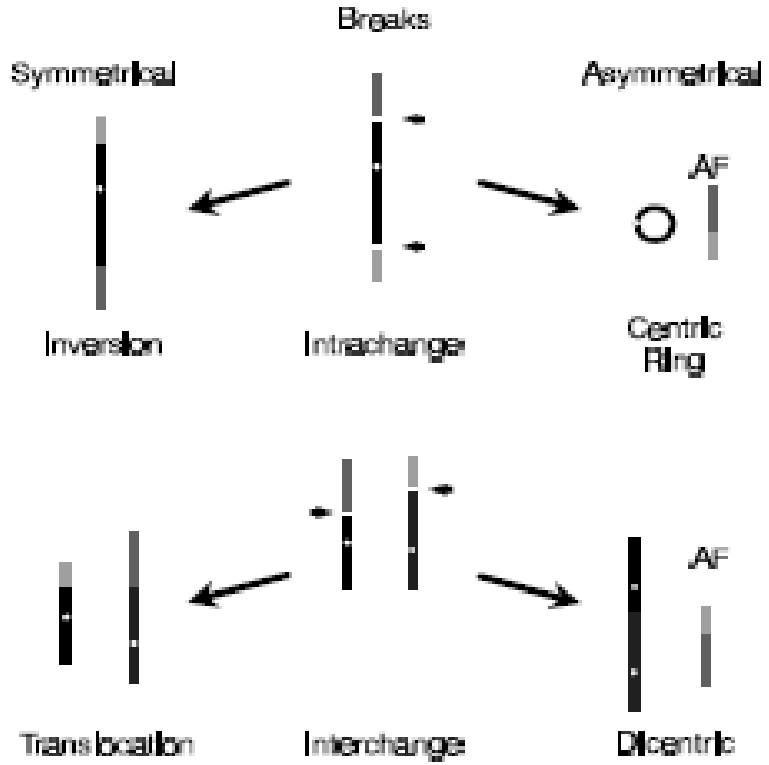
the limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body weight/day for treatment longer than 14 days
the first sampling interval is 1.5 normal cell cycle length (the latter being normally 12-18 hr)

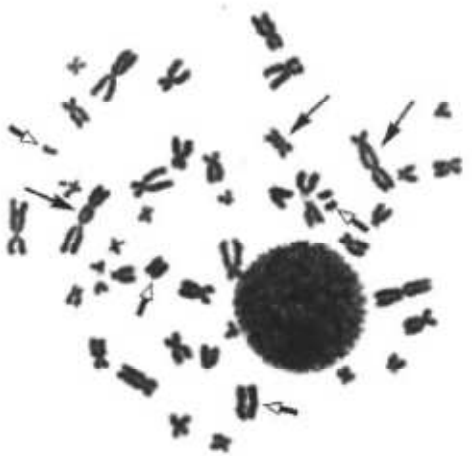
Chromosome preparations are made from the bone marrow cells and stained, and metaphase cells are analysed for chromosome aberrations.

Classification of Chromosomal Aberrations

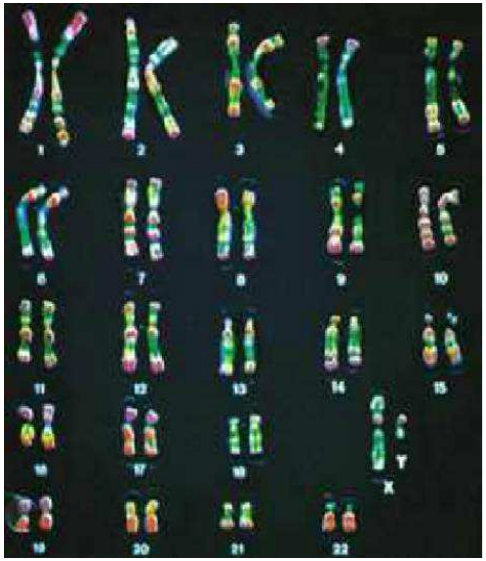
Definition for aberrations, including gaps:

- number of cells with chromosome aberrations
- type of chromosome aberrations given separately for each treated and control culture;





FISH Painting

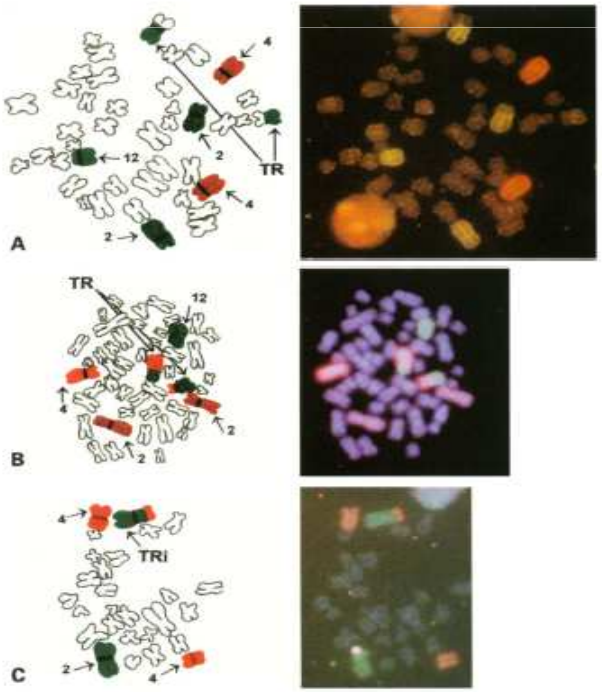


Fluorescent in situ hybridisation (FISH) method employs fluorescent-labelled probes for centromeres and chromosomes

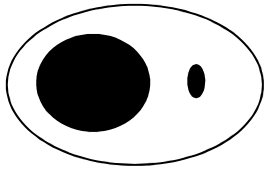
Generally painting three of the larger chromosomes (i.e. 1 → 12) representing about 20% of the genome leads to about 33% efficiency in detecting translocations.

Data obtained by the analysis of only a few chromosomes (the painted ones) generally are scaled up to full genomic frequency by assuming a random distribution of breaks points.

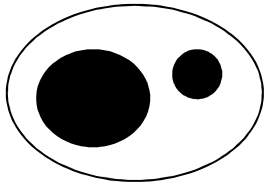
The estimation of the genomic translocation frequency is carried out using the formula of Lucas (1992) which links the observed translocat frequency on the painted chromosomes (F_p) to the genomic translocation frequency (F_g)

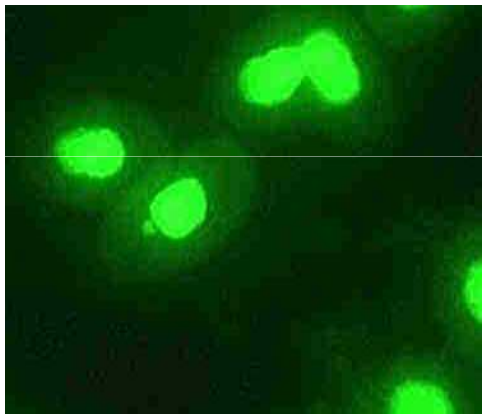


Micronucleus test

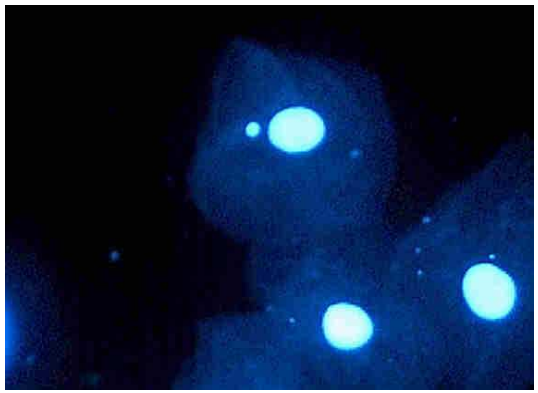


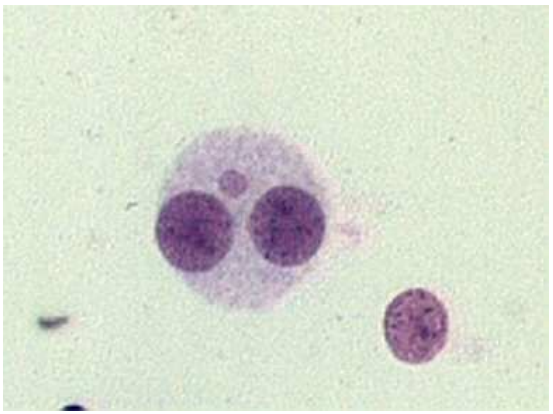
Micronuclei are the result of chromosome breakage and loss due to unrepaired or mis-repaired DNA lesions or chromosome malsegregation due to mitotic malfunction

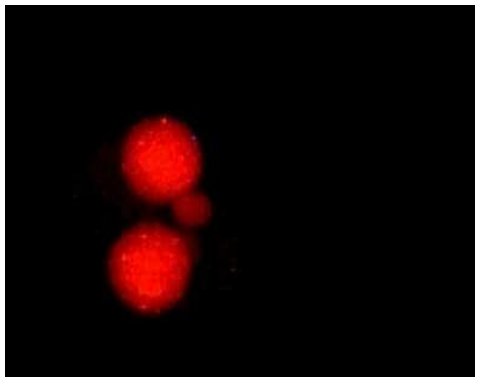




Micronucleus test can be applied in interphase to any proliferating cell populations regardless of its karyotype.







In Vitro Mammalian Cell Micronucleus Test OECD 487, 2010

- **Cultured primary human peripheral blood lymphocytes) or rodent cell lines such as CHO, V79, CHL/IU, and L5178Y cells may be used.**

- **The use of other cell lines and types should be justified based on their demonstrated performance in the assay. Cell types with a low, stable background frequency of micronucleus formation be used**

- **Cell cultures of human or mammalian origin are exposed to the test substance both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used.**

- **During or after exposure to the test substance, the cells are grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells.**

Cell cultures

Lymphocytes obtained from young (approximately 18-35 years of age), healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation : whole blood treated with an anti-coagulant (e.g. heparin), or separated lymphocytes, are cultured in the presence of a mitogen e.g. phytohaemagglutinin (PHA) prior to exposure to the test substance and cytoB

Established cell lines are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency in monolayers, and suspension cultures will not reach excessive density before the time of harvest, and incubated at 37°C.

Use of CytB

CytB should be used of as a cytokinesis blocker when human lymphocytes are used because cell cycle times will be variable within cultures and among donors and because not all lymphocytes will respond to PHA.

In studies with cytoB, cytostasis/cytotoxicity can be quantified from the cytokinesis-block proliferation index (CBPI) or may be derived from the RI from at least 500 cells per culture

Cytotoxicity

➢**In studies with cytB, cytostasis/cytotoxicity can be quantified from the cytokinesis-block proliferation index (CBPI) or may be derived from the RI from at least 500 cells per culture.**

➢**In studies without cytoB, it is necessary to demonstrate that the cells scored in the culture have undergone division during or following treatment with the test substance.**

Methods that have been used for ensuring that divided cells are being scored include:

✓ incorporation and subsequent detection of bromodeoxyuridine (BrdU) to identify cells that have replicated

✓the formation of clones when cells from permanent cell lines are treated and scored in situ on a microscope slide

✓(Proliferation Index (PI)) or the measurement of Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) or other proven methods.

Table 1. Cell treatment and harvest times for the MNvit assay

Lymphocytes, primary cells and cell lines treated with cytoB	+ S9	Treat for 3-6 hrs in the presence of S9; remove the S9 and treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
	– S9 Short exposure	Treat for 3-6 hrs; remove the treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
	– S9 Extended exposure	*Option A:* Treat for 1.5 – 2 normal cell cycles in the presence of cytoB; harvest at the end of the exposure period. *Option B:* Treat for 1.5 – 2.0 normal cell cycles; remove the test substance; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
Cell lines treated without cytoB (Identical to the treatment schedules outlined above with the exception that no cytoB is added) **For lymphocytes, the most efficient approach is to start the exposure to the test substance at 44-48 hrs after PHA stimulation, when cycle synchronisation will have disappeared**		

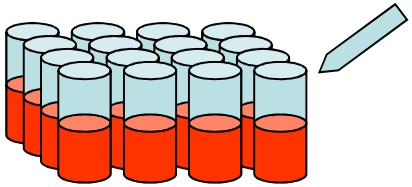
Cytokinesis-Block Micronucleus Assay

(I) PREPARATION OF CULTURES

Whole blood (0.4 ml) is added to complete medium RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillyn, 100 µg/ml streptomycin, 1,5 % phytoemoagglutinin.

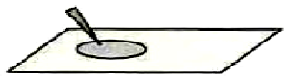
(II) ADDITION OF CYTOCHALASIN B

Cytochalasin B (6 µg/ml) is added.



(III) CELLS HARVESTING AND PREPARATION OF SLIDES

After 72 h cells are centrifuged, treated with hypotonic solution (0.075 M KCl) and then fixed in methanol and acetic acid 5:1.



(IV) SLIDE PREPARATION

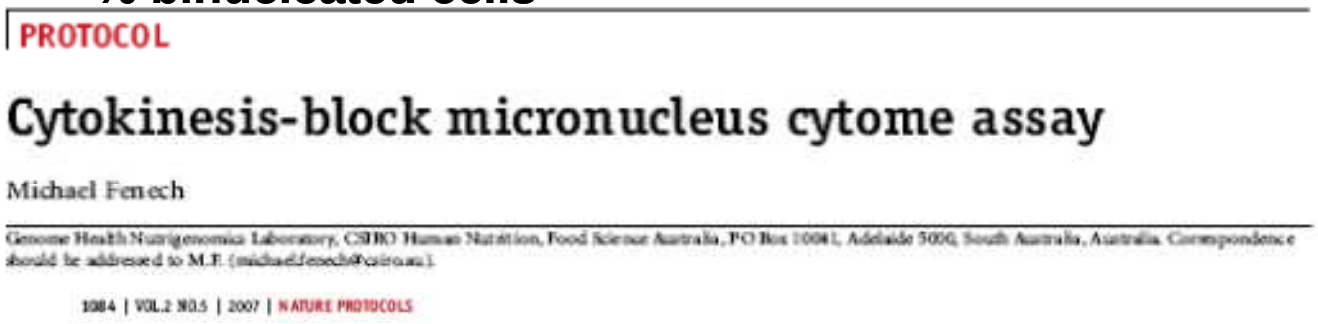
Lymphocytes are dropped onto clean iced slides, air dried and stained with Giemsa 3 %.

MICRONUCLEUS IN HUMAN LYMPHOCYTES/ESTABLISHED CELL LINES

Experimental protocol

Experimental protocol was standardized in isolated lymphocytes and in whole blood culture to evaluate:

- **Genotoxic damage**
- micronuclei (chromosome breakage and chromosome loss)
- Nucleoplasmic bridges
- Buds
- **Cytoxic effects**
- necrosis
- apoptosis
- **Cytostatic effects**
- Nuclear division index
- % binucleated cells



PROTOCOL

Cytokinesis-block micronucleus cytome assay

Michael Fenech

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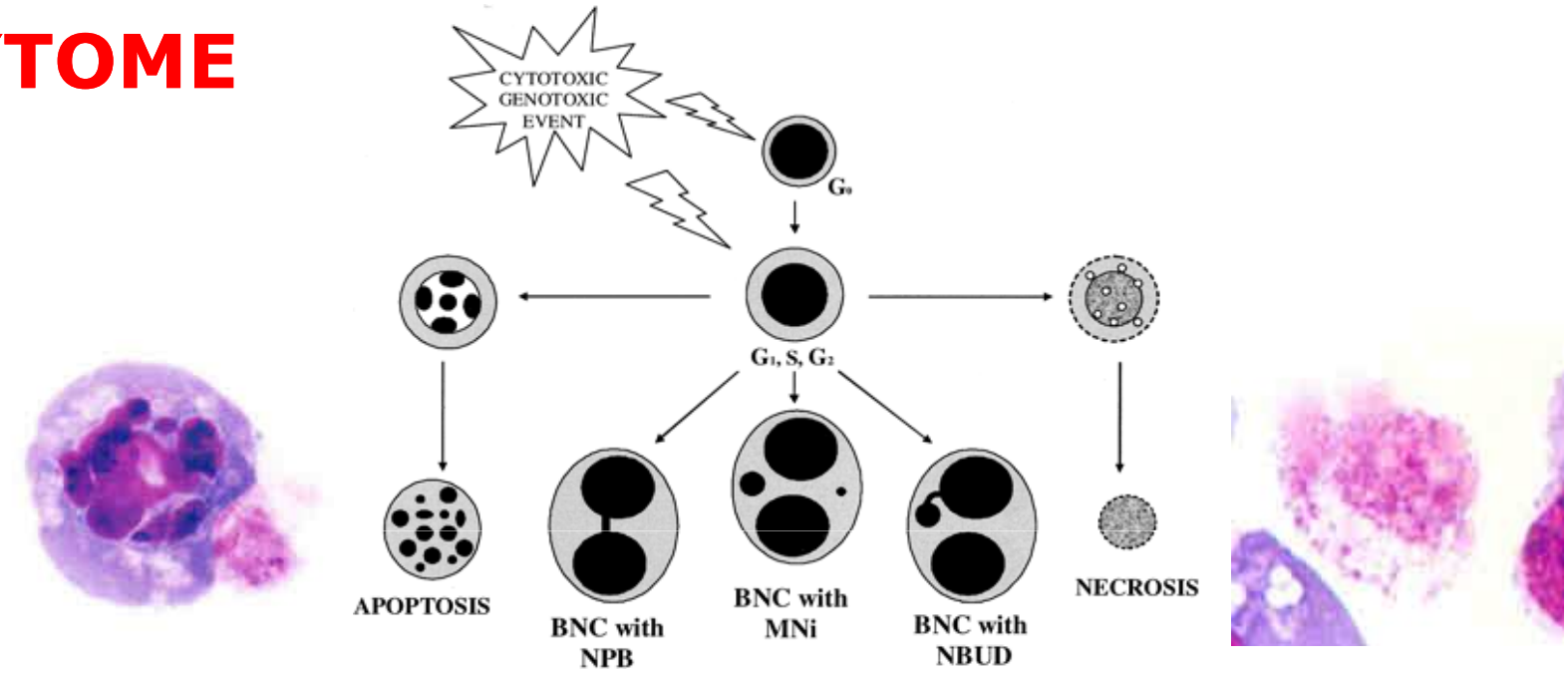
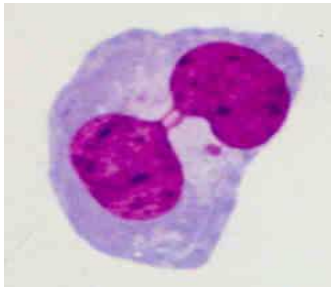
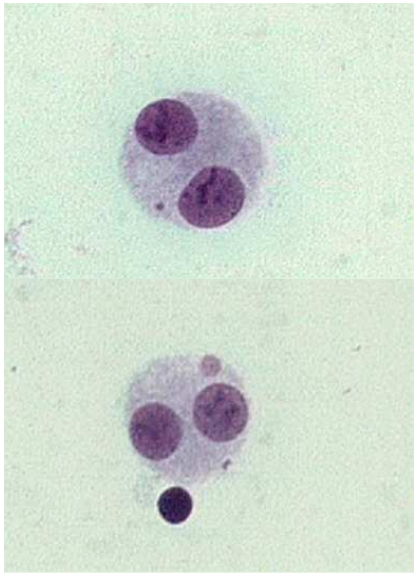
1084 | VOL.2 NO.5 | 2007 | NATURE PROTOCOLS

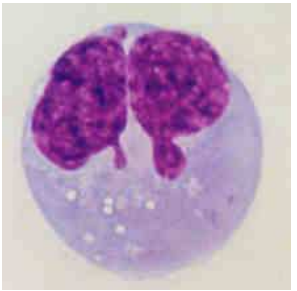
PERIPHERAL LYMPHOCYTES : CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY

CYTOME









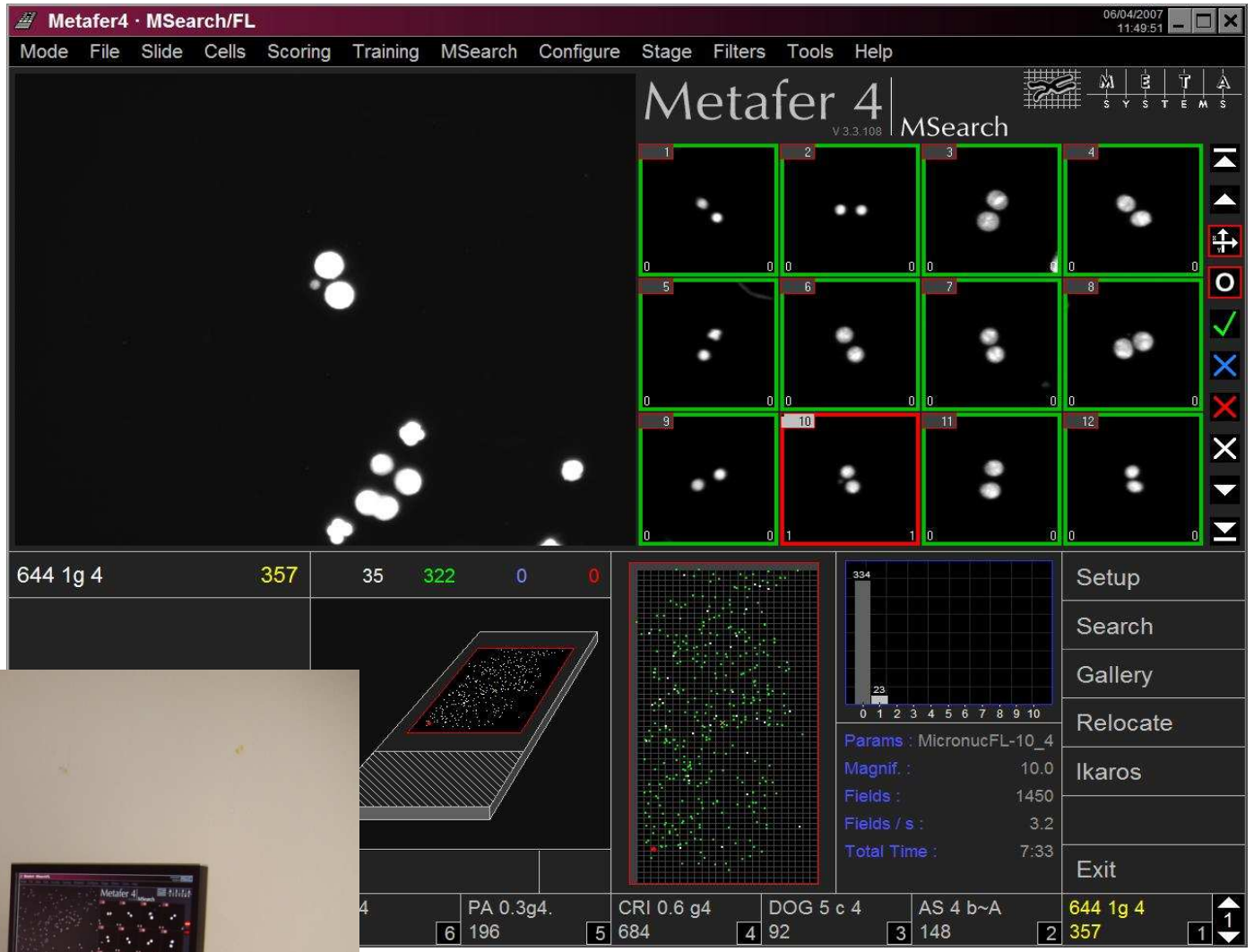
SCORING

- **In cytB-treated cultures, micronucleus frequencies should be analysed in at least 2000 binucleated cells per concentration (at least 1000 binucleated cells per culture; two cultures per concentration).**

$$\text{CBPI} = \frac{((\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

- **In cell lines assayed without cytoB treatment, micronuclei should be scored in at least 2000 cells per concentration (at least 1000 cells per culture; two cultures per concentration). Where only one culture per concentration is used, at least 2000 cells should be scored from that culture**

Automated scoring



Micronuclei,can be automatically detected with the *Metafer MSearch* micronucleus software module (*MicroNuclei*). Within a region of interest around a bi-nucleated cell the number of micronuclei is determined automatically.

Mammalian Erythrocyte Micronucleus Test
OECD 474 (1997)

Mice or rats are recommended if bone marrow is used, although any appropriate mammalian species may be used.

Animals are treated with the test substance once. Samples of bone marrow are taken at least twice, starting not earlier than 24 hours after treatment, but not extending beyond 48 hours after treatment with appropriate interval(s) between samples

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice.

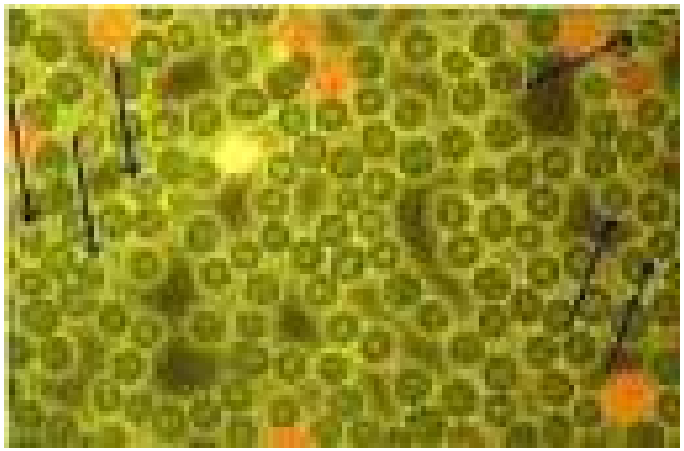
Mammalian Erythrocyte Micronucleus Test
OECD 474 (1997)

Analysis

The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood.

Flow cytometry

Microscopic analysis



Mutation Research 698 (2010) 30–37

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Community address: www.elsevier.com/locate/mutres

Evaluation of a liver micronucleus assay in young rats (III): A study using nine hepatotoxicants by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)–Mammalian Mutagenicity Study Group (MMS)

Hironao Takasawa[a,*], Hiroshi Suzuki[b], Izumi Ogawa[c], Yasushi Shimada[d], Kazuo Kobayashi[e], Yukari Terashima[e], Hirotaka Matsumoto[f], Chinami Aruga[g], Keiyu Oshida[h], Ryo Ohta[f], Tadashi Imamura[b], Atsushi Miyazaki[b], Masayoshi Kawabata[a], Shigenori Minowa[a], Makoto Hayashi[i]

Profiles of hepatotoxic agents and results of micronucleus assays in comparison with the published data of genotoxicity and carcinogenicity.

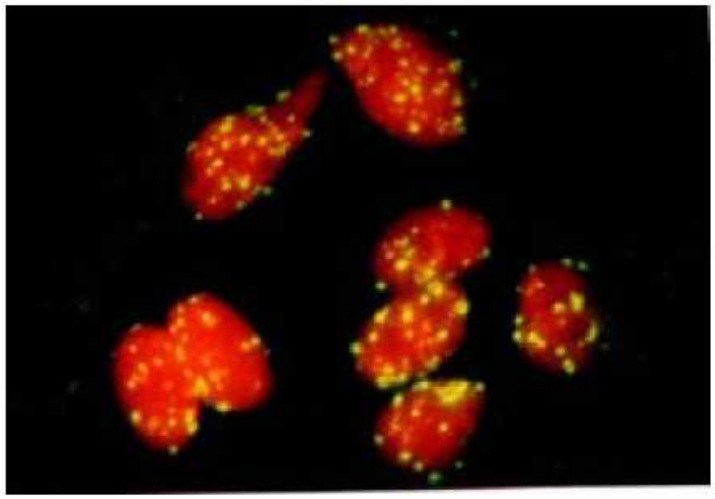
Test chemical	Result of MN		Genotoxicity			Carcinogenicity
	L	PB	Ames	*In vitro* CA	*In vivo* BM MN#	Target organ#
Necrotic compound						
2,6-Dinitrotoluene (2,6-DNT)	+	–	+ [18]	+ [18]	– [18]	+ L [18]
Bromobenzene (BBZ)	E	–	– [19]	– [19]/+[29]	(+ in mice) [19]	– [19]
Isoniazid	–	E	+ [26]	– [28]	ND	+ Lu [26,27]
Phenacetin	–	E	+ [58,59]	+ [26]	– [32]/+ in mice [32]	+ UB, K [34,35]
Allyl alcohol	–	–	– [38]	+ [38]	– [38]	– [36]
Thioacetamide (TAA)	–	–	– [60]	– [61]	ND	+ L [56,57]
Cholestatic compound						
Chlorpromazine HCl (CPZ)	–	±	– [23]	– [23]	+ [24,25]	+ P [22]
α-Naphthyl isothiocyanate (ANIT)	–	NT	– [39]	ND	ND	ND
Oxidative stress inducer						
Clofibrate	–	NT	– [62]	(–) [63]	ND	+ L, P [53–55]

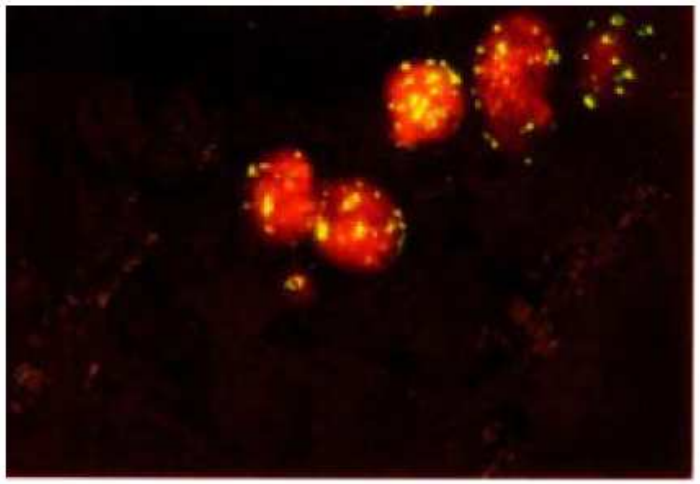
MN: micronucleus assay, L: liver, PB: peripheral blood, CA: chromosomal aberration assay (parentheses show *in vitro* MN result), BM: bone marrow (parentheses show PB MN result), NT: not tested, ND: no data found, P: pancreas, +: clear positive, –: negative, E: equivocal, ±: weakly positive, P: pancreas, Lu: lung, UB: urinary bladder, K: kidney, #: data in rats.

FISH MN

Aim:
Characterize micronuclei

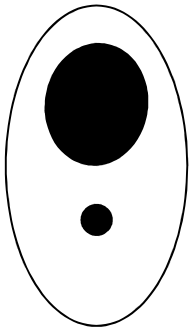
FISH was performed with directly labelled human pancentromeric and pantelomeric probes.

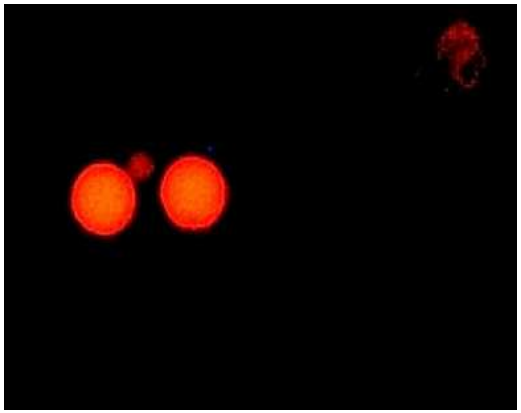


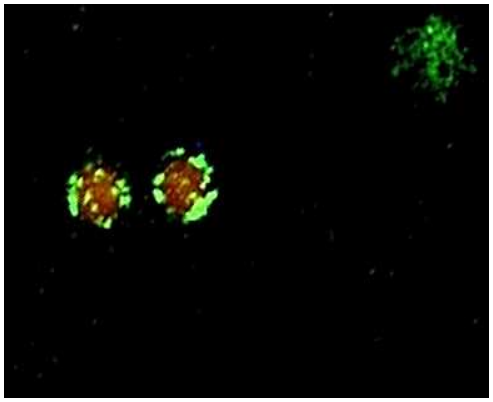


PERIPHERAL LYMPHOCYTES CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY

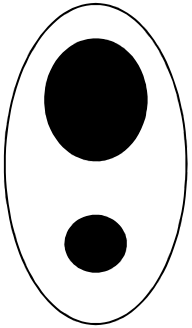
Characterization of MN after fluorescence *in situ* hybridization with a pan-centromeric probe

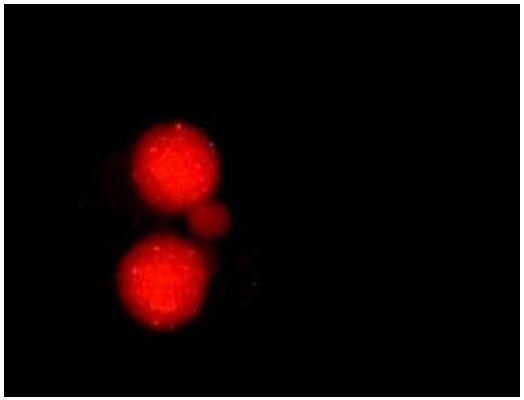


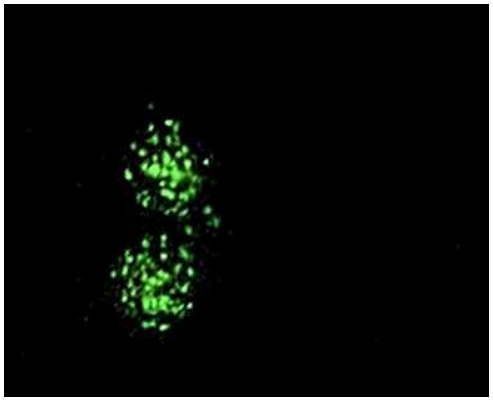




C-MN







C+MN

DNA DAMAGE TESTS

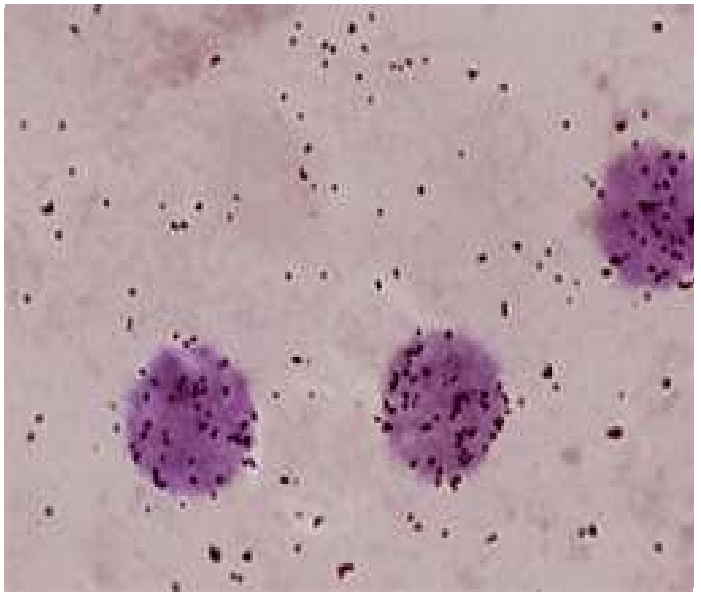
Unscheduled DNA Synthesis (UDS) Test in Mammalian Cells *in vitro*

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*
OECD 486 (1997)

COMET ASSAY

Unscheduled DNA synthesis (UDS)

This assay measures a cell's ability to perform global genomic nucleotide excision repair (NER).
Repair is quantified by the amount of radioactive thymidine incorporated after this insult, and the length of time allowed for this incorporation is specific for repair of particular lesions.



This can be detected by autoradiography associated with image analysis. This method involves culturing cells on glass slides, exposing them to the test substances in the presence of medium containing high specific radioactivity [3H]-thymidine and observing the radiolabel incorporated during UDS into those cells

Unscheduled DNA Synthesis (UDS) Test in Mammalian Cells *in vitro*

Primary mammalian hepatocytes, established cell lines and lymphocytes

Treatment

Cells are treated with the test substance in a medium containing 3H-TdR for an appropriate length of time.
At the end of the treatment period,medium should be removed from the cells, which are then rinsed, fixed and dried.

In order to discriminate between UDS and normal semi-conservative DNA replication,the latter may be reduced or inhibited, for example, by the use of an arginine-deficient medium,low serum content, or by hydroxyurea in the culture medium.

Autoradiographic determinations

At least 50 cells per culture should be counted determining UDS, S-phase nuclei are not counted.

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo* OECD 486 (1997)

Aim of the test:

to identify substances that induce DNA repair in liver cells of treated animals

The endpoint of unscheduled DNA synthesis (UDS) is measured by determining the uptake of labelled nucleosides in cells that are not undergoing scheduled (S-phase) DNA synthesis.

uptake of tritium-labelled thymidine (3H-TdR) by autoradiography.

The detection of a UDS response is dependent on the number of DNA bases excised and replaced at the site of the damage.

Limitations:
UDS test detect mainly substance-induced "longpatch repair" (20-30 bases) NER pathway

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Procedure:

Treatment *in vivo* by gavage

Preparation of liver cells

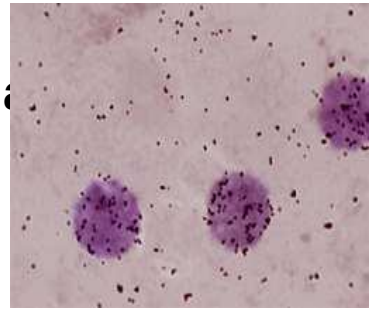
Liver cells are prepared by perfusion from treated animals normally 12-16 hours after dosing

Determination of UDS

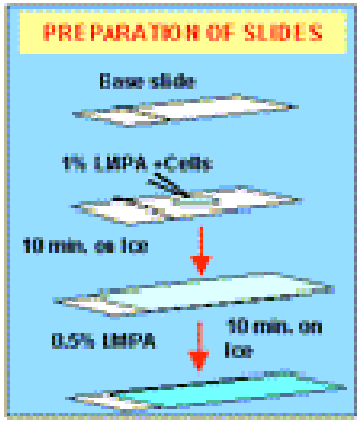
Freshly isolated mammalian liver cells are incubated in vitro usually with medium containing 3HTdR for an appropriate length of time, e.g. 3 - 8 hours

Results

- **number of "cells in repair" if determined;**
- **number of S-phase cells if determined;**
- **historical negative (solvent/vehicle) and positive control da with range, means and standard deviations**

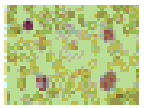


DNA DAMAGE



Single Cell Suspension

DNA Rejoining - Repair



Cells, tissues, cell line, etc.

Preparation of Slides

Neutral Lysis

Alkaline Lysis

2hrs

COPLIN JAR FOR LYSIS

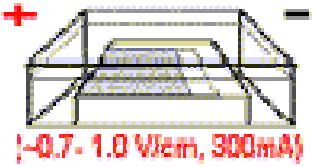
Alkaline Unwinding

Alkaline Unwinding

Double Strand Break Detection

Single Strand Break Detection

Neutral Wash



(~0.7- 1.0 V/cm, 300mA)

Alkaline Electrophoresis

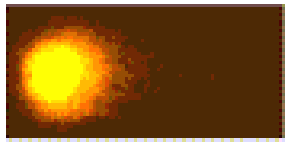
Neutral Electrophoresis

Neutralize
(0.4 M Tris, pH7.5)

5 min., three times

Image Processed

Stain Slide
(75µl EtBr, 20 µg/ml)



The comet assay

Lymphocytes in
1% agarose on
microscope slide

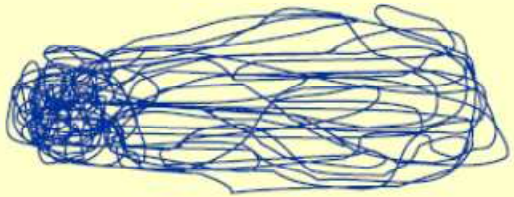
Lysis: Triton X-100, 2.5 M NaCl

Nucleoid; supercoiled DNA

Alkaline incubation:
0.3 M NaOH, 10 mM EDTA

Electrophoresis: 0.8 V/cm, 30 min

Neutralisation, DAPI stain,
fluorescence microscopy



Broken DNA loops extend towards anode, forming tail of comet

% of DNA in tail is related to DNA break frequency

CRITICAL STEPS

- **Isolation of cells. Most animal cells can be used if it is possible to disaggregate them to release single cells**
- **Embedding cells**
- **Lysis of cells**
- **Alkaline treatment and electrophoresis**
- **Ways of measuring comets:**
  - **manual scoring on a photomicrograph**
  - **Visual scoring**
  - **Image analysis**

Image analysis:

% DNA in tail?

Tail moment?

Tail length?

- '% DNA in tail' is linearly related to DNA break frequency over wide range of damage.
- 'Tail length' increases only over very low range of breaks.
- 'Tail moment' combines these measures and is non-linear.
- 'Tail moment' has no recognised units and is impossible to visualise.

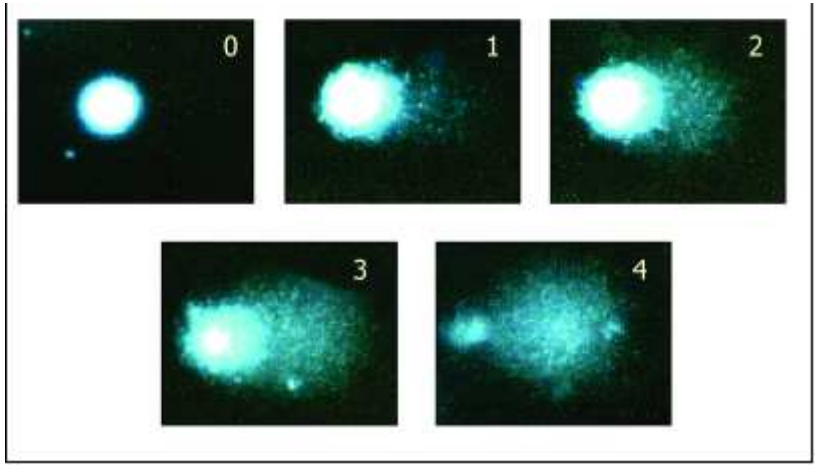
USE % DNA IN TAIL!

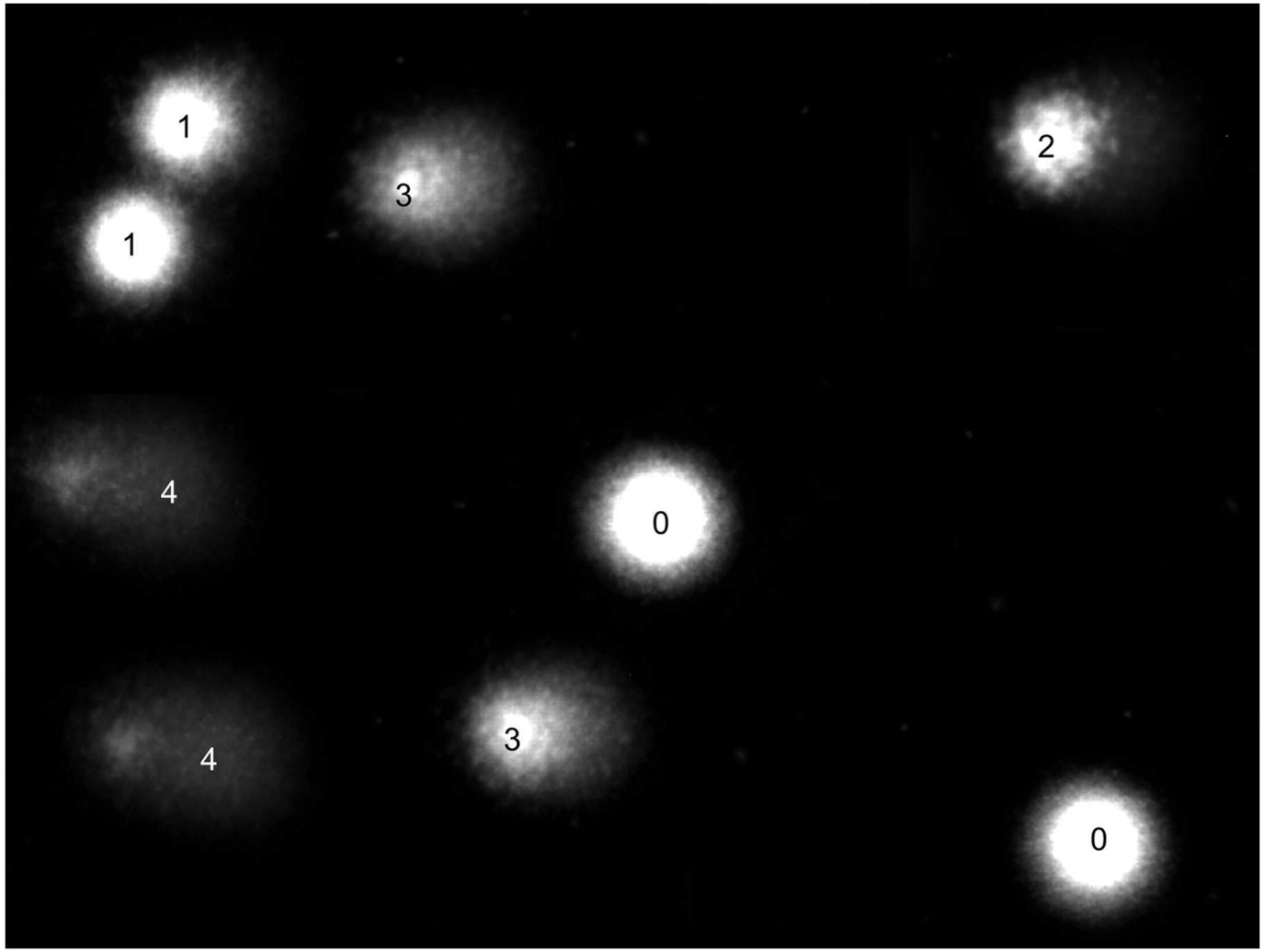
Ways of measuring Comet

Visual scoring

Comets are selected for scoring

Overlapping comet cannot be scored with image analysis





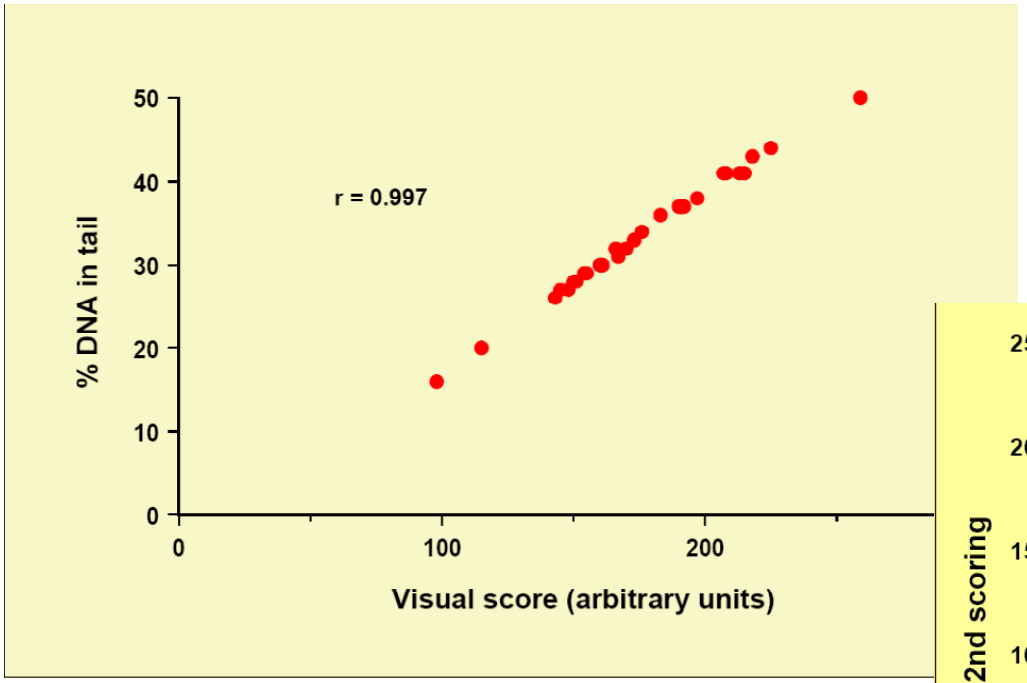
Collins, A. R. et al. Mutagenesis 2008 23:143-151; doi:10.1093/mutage/gem051

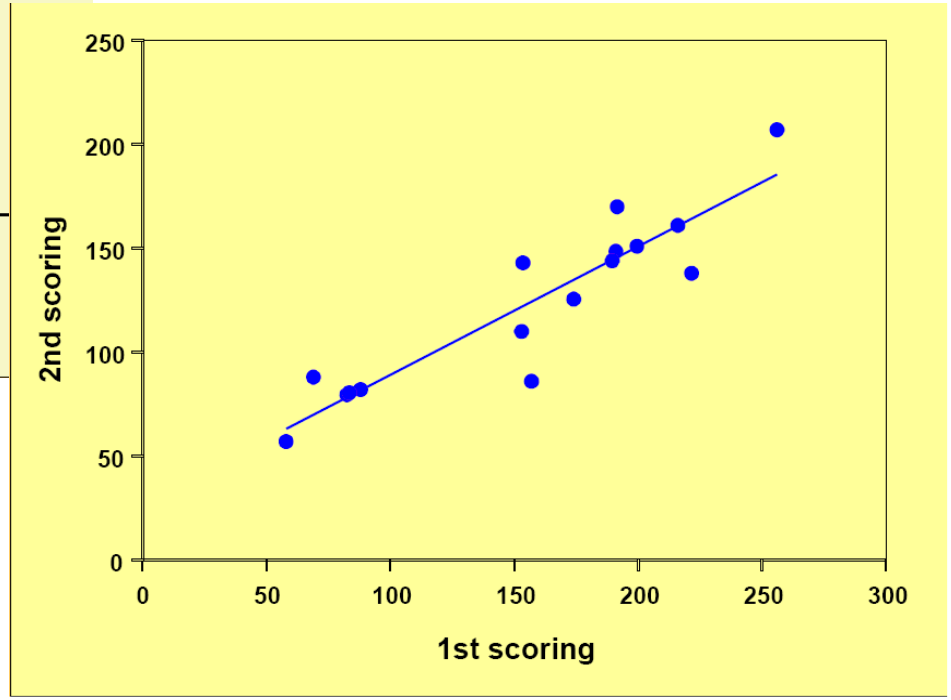
mutagenesis

Ways of measuring Comet

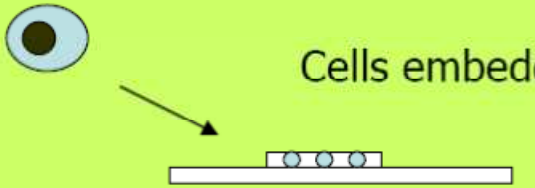
Visual scoring vs image analysis

Correlation and reproducibility





Detecting DNA damage using lesion-specific enzymes



Cells embedded in agarose on microscope slide

Lysis: Triton X-100, 2.5 M NaCl

Nucleoid;
supercoiled DNA

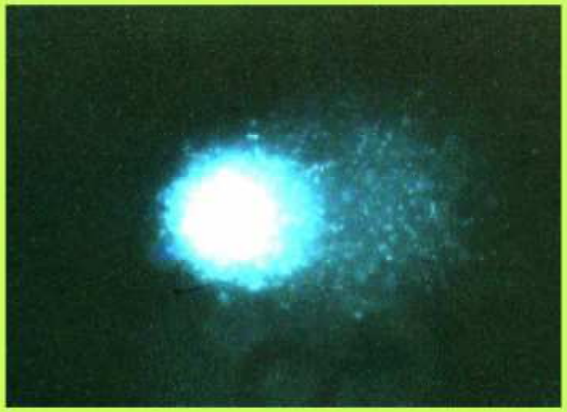
± Digestion with lesion-specific endonuclease (FPG, endonuclease III)

Alkaline incubation:
0.3 M NaOH, 10 mM EDTA

Electrophoresis: 0.8 V/cm, 30 min

Neutralisation, DAPI stain, fluorescence microscopy

The frequency of damaged bases is given by the increase in DNA breaks in the presence of the specific endonuclease

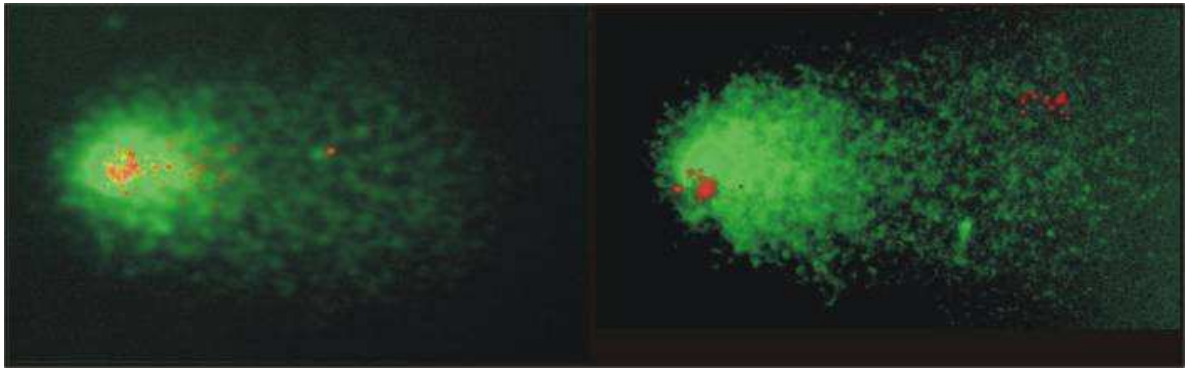


This assay allows us to estimate the level of oxidative base damage in lymphocytes

The enzymes; repair glycosylases (endonucleases)

- Formamidopyrimidine DNA glycosylase (FPG) recognises ring-opened purines (fapy-adenine, fapy-guanine) and 8-oxoguanine; the latter is thought to be its main substrate *in vivo.*
- Endonuclease III detects oxidised pyrimidines.
- AlkA detects 3-methyladenine (but is not very specific)
- T4 endonuclease V detects UV-induced pyrimidine dimers
- Uracil DNA glycosylase (misincorporated U)
- UvrABC? (Bulky adducts, helix distortions) – Not yet working satisfactorily

FISH COMET



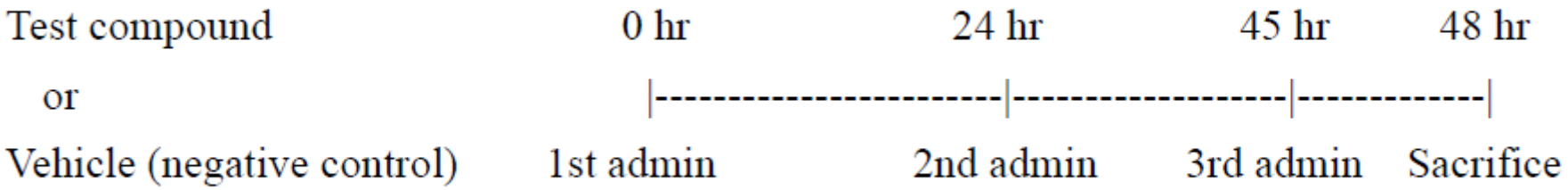
COMET ASSAY IN VIVO

Experimental design

Compound	Dose (mg/kg/day)	Number of animals (see note 1)
Vehicle (negative control)	0	5
EMS (positive control)	200	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

COMET ASSAY IN VIVO

Treatment schedule



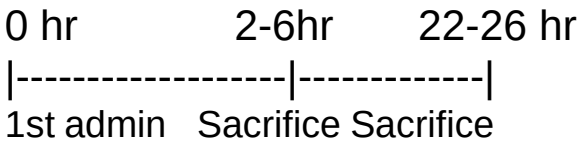


Table I. Common *in vitro* bacterial assays

Assay	Strain	End point	Comments	Published guidelines	References
Salmonella typhimurium reverse mutation assay	TA1535, TA1537 (or TA97 or TA97a), TA98, TA100	Primarily detects G/C base pair and frameshift mutations	Contain specific mutations in one of several genes involved in histidine biosynthesis that must be reverted to function normally. Testing with and without appropriate exogenous metabolic activation system. May not detect some oxidizing mutagens and cross-linking agents.	OECD Test Guideline 471 (replaces old OECD Test Guidelines 471 and 472)	(17–19)
S.typhimurium	TA102	Primarily detects A/T base pair damage and small deletions	Detects oxidizing mutagens and cross-linking agents	OECD Test Guideline 471	(19,20)
Other *S.typhimurium* mutants	YG1021, YG1026 (NR overexpression); YG1024, YG1029 (NAT overexpression)		For detection of mutagenicity of nitroaromatic and aminoaromatic substances that are bioactivated by NR and NAT. More sensitive than conventional strains. Used for detecting mutagenicity of toxic pollutants in air, water and food.		(21,22)
Escherichia coli reverse mutation assay	WP2, WP2*uvrA*	Primarily detects A/T base pair damage	Detects oxidizing mutagens and cross-linking agents	OECD Test Guideline 471	(19)

A, adenine; C, cytosine; G, guanine; NAT, *N*-acetyltransferase; NR, nitroreductase; T, thymine.

Table II. Common *in vitro* mammalian assays

Assay	Method/end point	Main attributes	Comments	Published guidelines	References
Mouse lymphoma TK gene mutation assay	L5178Y mouse lymphoma cell line; using a selective medium, mutant frequencies are determined	Detects not only point mutations but also various sizes of chromosome deletions and other effects that can lead to loss of heterozygosity (e.g. mitotic recombination, gene conversion and translocations)	Use of positive controls and colony sizing essential for quality control. Evaluation and interpretation changed over the years. Recent protocol updates recommendations. Can be used as alternative to metaphase analysis.	OECD Test Guideline 476; IWGT guidelines	(3,23–26)
HPRT gene mutation assay	Chinese hamster ovary, AS52 or other suitable cell line; using a selective medium, mutant frequencies are determined	Detects not only point mutations but also small deletions; larger deletions may be detected in AS52 cells	Use of positive controls essential for quality control	OECD Test Guideline 476	(23,27)
Metaphase analysis (*in vitro* mammalian chromosome aberration test)	A metaphase-arresting substance (e.g. colchicine) is applied; metaphase cells are analysed for the presence of structural chromosome aberrations	Detects clastogenicity; some information on aneugenicity can be obtained with extended culture times	A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes) (28)	OECD Test Guideline 473	(29–31)
Micronucleus test	Detects micronuclei in the cytoplasm of cultured mammalian cells during interphase	Detects both aneugenic and clastogenic substances; established mammalian lines, cultured human peripheral blood lymphocytes or Syrian hamster embryo cells may be used	Several developments in updating the protocol. Immunochemical labelling of kinetochores or hybridization with general or chromosome-specific centromeric/telomeric probes gives information on the nature and mechanism of formation of micronuclei induced (whole chromosomes or fragments).	Draft OECD Test Guideline 487	(13,32–36)

Table III. Common *in vivo* genotoxicity assays

Assay	End point	Main attributes	Comments	Published guidelines	References
Micronucleus test in erythropoietic cells	Structural and numerical chromosome alterations	Long history, regulatory acceptance, high relevance of end point	Has potential for application to other tissues	OECD Test Guideline 474	(15,28), and references cited therein
Metaphase analysis *in vivo*	Structural and numerical chromosome aberrations	Long history, regulatory acceptance, high relevance of end point	Has potential for application to other tissues	OECD Test Guideline 475	(39)
Transgenic animal models	Gene mutation	Can be applied to many tissues. Gene specific. No selective pressure on mutations. Relevant end point.	Need to optimize protocols overall and for each tissue. *lacI*, *lacZ*, *gpt* systems not sensitive to the detection of large deletions. Spi^- system detects large deletions.	IWGT, IPCS guidance	(40–44)
Chemically modified DNA	Covalent DNA adducts, oxidative lesions (e.g. 8-OH-dG)	Can be applied to many tissues. Can be highly sensitive (^{32}P-postlabelling or AMS) or chemically specific (MS). Other methods include immunochemical techniques, fluorescence, ECD (for 8-OH-dG).	Indicator test detecting premutagenic lesions. Interpretation of results can be complicated.	IWGT guidance	(45)
DNA strand breakage assays (e.g. comet assay)	DNA strand breaks, alkali-labile lesions	Can be applied to many tissues. Incorporation of enzymes can improve specificity. Cell division not required.	Indicator tests. Need to optimize protocols for different tissues. May be unable to detect mutagens that do not induce strand breaks or alkali-labile lesions, but may detect repair-induced breaks. Apoptosis/necrosis need to be controlled.	IWGT guidance	(14,46–49)
Liver UDS	Thymidine incorporation outside S phase	Long history of use; useful for some classes of substances.	Indicator test detecting repair activity. Uncertain acceptability and questionable sensitivity. Limited use in other tissues.	OECD Test Guideline 486	(50,51)

8-OH-dG, 8-hydroxy-2′-deoxyguanosine; AMS, accelerator mass spectrometry; ECD, electrochemical detection; MS, mass spectrometry; UDS, unscheduled DNA synthesis.

Table IV. Germ cell assays

Assay	End point	Main attributes[a]	Comments	Published guidelines	References
Class 1: tests in germ cells *per se*					
Transgenic animal models	Gene mutation	Gene specific. No selective pressure on mutations. Relevant end point.	See Table III	See Table III	See Table III
ESTR assay	Non-coding tandem repeat DNA mutation	Potentially relevant end point. Detects heritable mutations at ambient exposure levels. Uses relatively few animals. Can be conducted in humans.	Some tandem repeat mutations also occur in, or near, coding genes. Although there are parallels with mutations in coding genes, the human health outcomes require further study.		(52–55)
Mammalian spermatogonial chromosome aberration test	Structural chromosome aberrations	Relevant end point		OECD Test Guideline 483	(56)
FISH assays	Structural chromosome aberrations; sperm aneuploidy	Relevant end points. Can be conducted in humans.	See Table III	See Table III	(57,58)
Comet assay	DNA strand breaks or alkali-labile sites	See Table III. Can be conducted in humans.	See Table III	See Table III	(59)
Chemically modified DNA	DNA adducts	See Table III. Can be conducted in humans.	See Table III	See Table III	(60)
Class 2: tests to detect effects in the offspring (or potential offspring)					
ESTR assay	As above for class 1 tests	As above for class 1 tests	As above for class 1 tests		As above for class 1 tests
Dominant lethal test	Reduction in viable embryos attributed to chromosome or gene mutations	Relevant end point. Provides data for quantification of pregnancy loss.		OECD Test Guideline 478	(61)
Mouse visible specific locus test	Gene mutation	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	EPA OPPTS 870.5200	(62)
Mouse biochemical specific locus test	Gene mutation	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	EPA OPPTS 870.5195	(63)
Mouse heritable translocation assay	Structural chromosome aberrations	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	OECD Test Guideline 485	(64)

EPA OPPTS, United States Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances; ESTR, Expanded Simple Tandem Repeat; FISH, fluorescence *in situ* hybridization.

[a]'Relevant end point' means relevant to the estimation of human heritable health risk.

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

- Bacterial Reverse Mutation Test
- *In Vitro* Mammalian Chromosome Aberration Test
- Mammalian Erythrocyte Micronucleus Test
- Mammalian Bone Marrow Chromosome Aberration Test
- *In Vitro* Mammalian Cell Gene Mutation Test
- Genetic Toxicology: Sex-linked Recessive Lethal Test in Drosophila Melanogaster
- Genetic Toxicology: Rodent Dominant Lethal Test
- Genetic Toxicology: *in Vitro* Sister Chromatid Exchange Assay in Mammalian Cells
- Genetic Toxicology: *Saccharomyces Cerevisae*, Gene Mutation Assay
- Genetic Toxicology: *Saccharomyces Cerevisae*, Miotic Recombination Assay
- Genetic Toxicology: DNA Damage And Repair, Unscheduled DNA Synthesis in Mammalian Cells *in Vitro*
- Mammalian Spermatogonial Chromosome Aberration Test
- Genetic Toxicology: Mouse Spot Test
- Genetic Toxicology: Mouse Heritable Translocation Assay
- Unscheduled DNA Synthesis (USD) Test With Mammalian Liver Cells *in Vivo*

Performance of genotoxicity tests to predict rodent carcinogenicity

	Carcinogens	Non-carcinogens
Genotoxicity positive	A	B
Genotoxicity negative	C	D

Sensitivity	% correctly identified carcinogens	A/(A+C) * 100
Specificity	% correctly identified non-carcinogens	D/(B+D) * 100
Concordance	% correctly identified carcinogens and non-carcinogens	(A + D)/(A+B+C+D) * 100
Positive predictivity	% carcinogens among positive genotoxicity results	A/(A+B) *100
Negative predictivity	% non-carcinogens among negative genotoxicity results	D/(C+D) * 100

Performance of *in vitro* genotoxicity tests in detecting rodent carcinogens

	MN	MLA	Ames	CA
N	115	350	717	488
Sensitivity	78.7	73.1	58.8	65.6
Specificity	30.8	39.0	73.9	44.9
Positive predictivity	79.5	73.7	87.4	75.5
Negative predictivity	29.6	38.3	36.8	33.5

Sensitivity=TP/TP + FN
Specificity =TN/FP +TN
Positive predictivity= %carcinogens/total positive compounds
Negative predictivity= % non carcinogens/total negative compounds

Performance of *in vivo* genotoxicity tests in detecting rodent carcinogens

	CA Kim and Margolin	MN Kim and Margolin	UDS Lambert	Comet Sasaki
N	82	82	37	190
Sensitivity	43.6	36.4	69.0	78.1
Specificity	66.7	77.8	60.0	80.0
Positive predictivity	72.7	76.9	92.0	95.4
Negative predictivity	36.7	37.5	23.0	40.7

Sensitivity=TP/TP + FN
Specificity =TN/FP +TN
Positive predictivity= %carcinogens/total positive compounds
Negative predictivity= % non carcinogens/total negative compounds

Genotoxicity testing

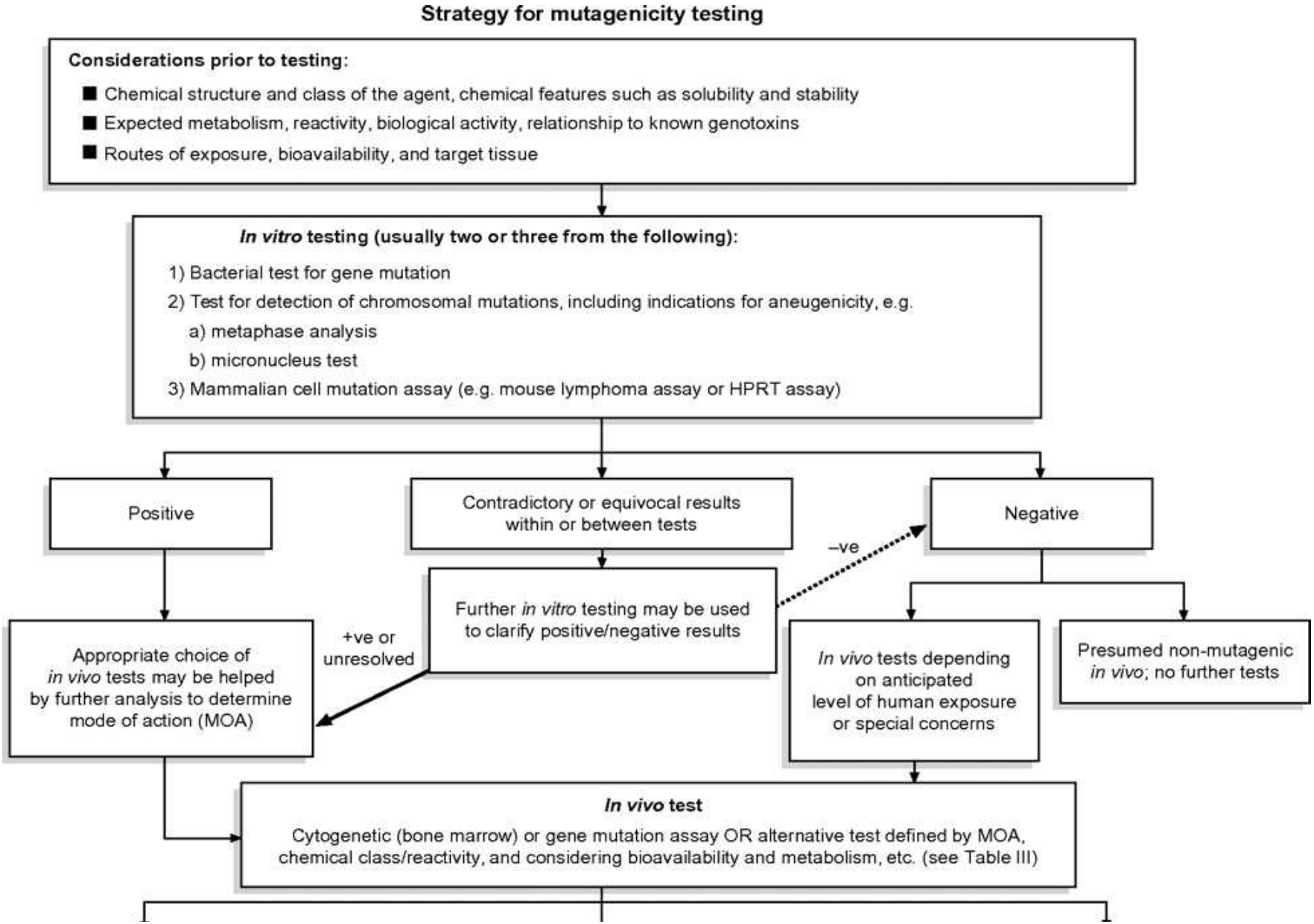
An adequate evaluation of the genotoxic potential of a chemical substance involve the assessment of multiple genetic endpoints:

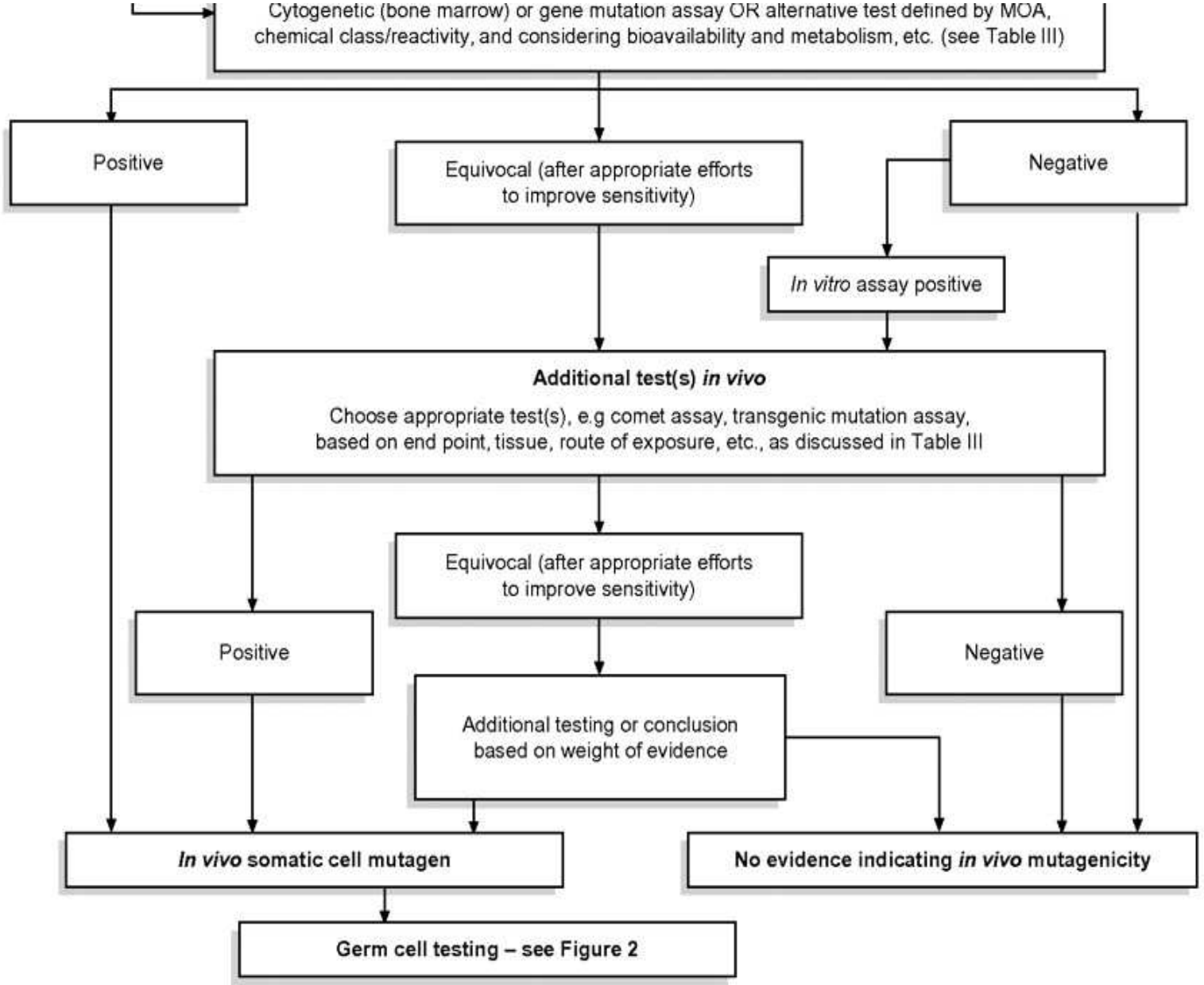
- **Gene mutation**
- **Structural Chromosomal alterations**
- **Numerical Chromosomal alterations**

An adequate coverage of all the genetic endpoints can only be obtained by the use of multiple test systems : a test battery.

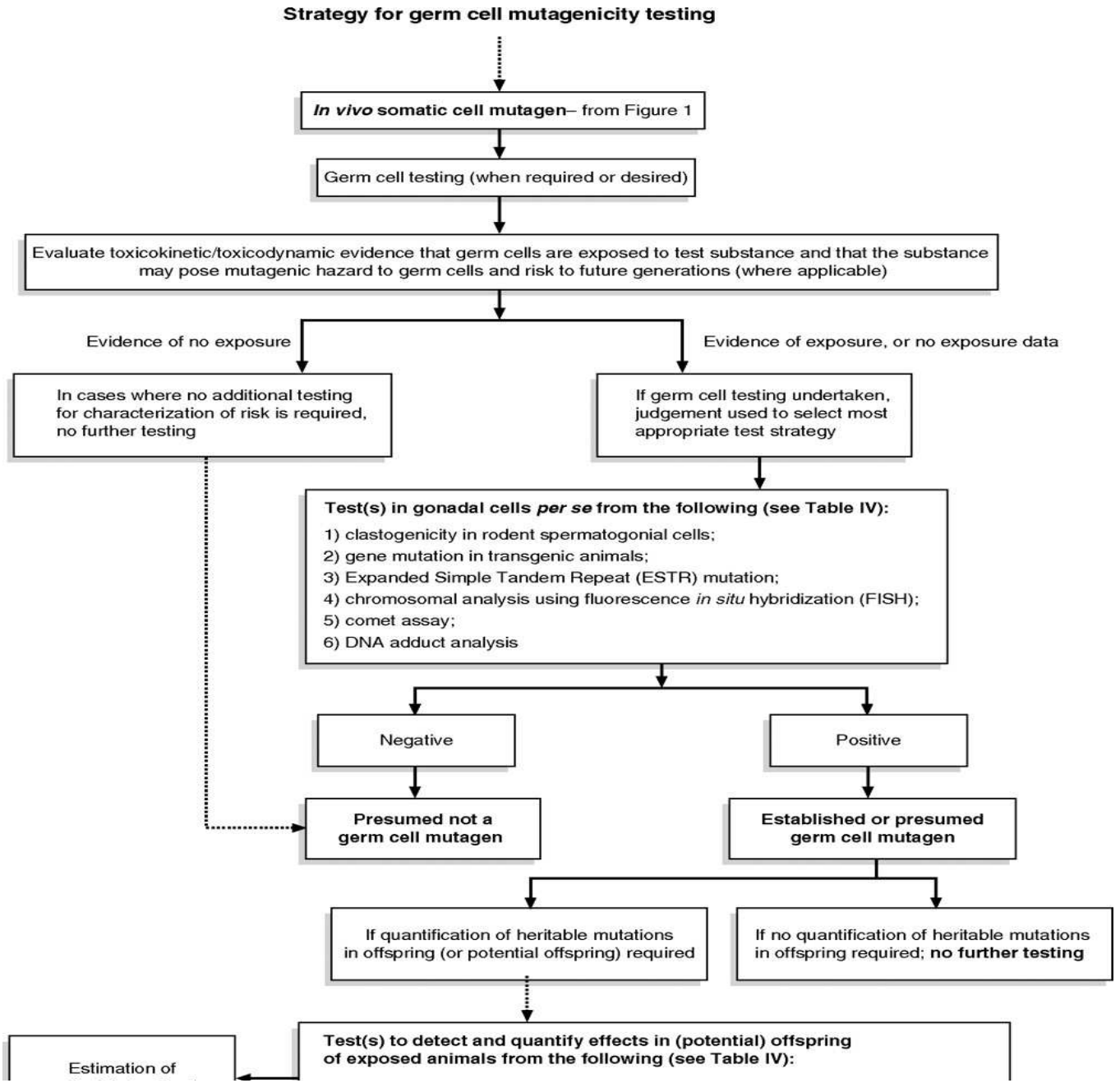
No individual test can simultaneously provide information on all end points.

WHO/IPCS HARMONIZED SCHEME




Cytogenetic (bone marrow) or gene mutation assay OR alternative test defined by MOA, chemical class/reactivity, and considering bioavailability and metabolism, etc. (see Table III)
Positive
Equivocal (after appropriate efforts to improve sensitivity)
Negative
In vitro assay positive
Additional test(s) in vivo
Choose appropriate test(s), e.g comet assay, transgenic mutation assay, based on end point, tissue, route of exposure, etc., as discussed in Table III
Equivocal (after appropriate efforts to improve sensitivity)
Positive
Negative
Additional testing or conclusion based on weight of evidence
In vivo somatic cell mutagen
No evidence indicating in vivo mutagenicity
Germ cell testing – see Figure 2

WHO/IPCS HARMONIZED SCHEME



Scientific Committee on Consumer Safety

SCCS

THE SCCS'S NOTES OF GUIDANCE FOR THE TESTING OF COSMETIC INGREDIENTS AND THEIR SAFETY EVALUATION

7TH REVISION

The SCCS adopted this opinion at its 9th plenary meeting
of 14 December 2010.

IN VITRO CORE BATTERY

- **test for gene mutation in bacteria**
  Ames

- **Test for gene mutation in mammalian cells**

- **cytogenetic test for structural and numerical chromosomal damage**
  metaphase chromosome aberration test or
  micronucleus test

Generation and evaluation of data on genotoxic potential

A step-wise approach involves:

- **a basic battery of *in vitro tests***

- **consideration of whether specific features of the test substance might require substitution of one or more of the recommended in vitro tests by other in vitro or in vivo tests in the basic battery**

- **in the event of positive results from the basic battery, review of all the available relevant data on the test substance**

- **- where necessary, conduct of an appropriate in vivo study (or studies) to assess whether the genotoxic potential observed in vitro is expressed in vivo.**

REGOLAMENTO (CE) n. 1223/2009 DEL PARLAMENTO EUROPEO E DEL CONSIGLIO del 30 novembre 2009 sui prodotti cosmetici

(9) I prodotti cosmetici dovrebbero essere sicuri nelle condizioni normali o ragionevolmente prevedibili di uso. In particolare, i rischi per la salute umana non dovrebbero essere giustificati attraverso un'analisi rischi-benefici.

Limitazioni di talune sostanze

Il regolamento vieta l'impiego delle sostanze classificate come cancerogene, mutagene o tossiche per la riproduzione (CMR), salvo casi eccezionali. Prevede un elevato livello di protezione della salute umana in caso di impiego di nanomateriali nei prodotti cosmetici.

Gli allegati al presente regolamento riportano un elenco di sostanze il cui impiego è vietato (allegato II) o limitato (allegato III) nei prodotti cosmetici. Sono altresì vietati alcuni coloranti (diversi da quelli dell'allegato IV), conservanti (diversi da quelli dell'allegato V) e filtri UV (diversi da quelli dell'allegato VI).

Tabella 3.6.1

Categorie di pericolo per le sostanze cancerogene

Categorie	Criteri
CATEGORIA 1:	Sostanze cancerogene per l'uomo accertate o presunte La classificazione di una sostanza come cancerogena di categoria 1 avviene sulla base di dati epidemiologici e/o di dati ottenuti con sperimentazioni su animali. La classificazione di una sostanza come cancerogena di:
Categoria 1A:	categoria 1 A può avvenire ove ne siano noti effetti cancerogeni per l'uomo sulla base di studi sull'uomo, oppure di
Categoria 1B:	Categoria 1B per le sostanze di cui si presumono effetti cancerogeni per l'uomo, prevalentemente sulla base di studi su animali. La classificazione di una sostanza nelle categorie 1A e 1B si basa sulla forza probante dei dati e su altre considerazioni (cfr. punto 3.6.2.2). I dati possono provenire da: — studi condotti sull'uomo da cui risulta un rapporto di causalità tra l'esposizione umana a una sostanza e l'insorgenza di un cancro (sostanze di cui sono accertati effetti cancerogeni per l'uomo); o — sperimentazioni animali i cui risultati (1) permettono di dimostrare effetti cancerogeni per gli animali (sostanze di cui si presumono effetti cancerogeni per l'uomo). Inoltre, caso per caso, in base a una valutazione scientifica può essere deciso di considerare una sostanza come presunta sostanza cancerogena se esistono studi che dimostrano la presenza di effetti cancerogeni limitati per l'uomo e per gli animali.
CATEGORIA 2:	Sostanze di cui si sospettano effetti cancerogeni per l'uomo La classificazione di una sostanza nella categoria 2 si basa sui risultati di studi sull'uomo e/o su animali non sufficientemente convincenti per giustificare la classificazione della sostanza nelle categorie 1A o 1B, tenendo conto della forza probante dei dati e di altre considerazioni (cfr. punto 3.6.2.2). Tali dati possono essere tratti da studi che dimostrano la presenza di effetti cancerogeni limitati (1) per l'uomo o per gli animali.

(1) Nota: Cfr. 3.6.2.2.4.

Categoria di rischio Categorie di pericolo per le sostanze mutagene delle cellule germinali

Categorie	Criteri
CATEGORIA 1:	Sostanze di cui è accertata la capacità di causare mutazioni ereditarie o da considerare come capaci di causare mutazioni ereditarie nelle cellule germinali umane. Sostanze di cui è accertata la capacità di causare mutazioni ereditarie nelle cellule germinali umane.
Categoria 1A:	La classificazione nella categoria 1A si basa su risultati positivi di studi epidemiologici sull'uomo. Sostanze da considerare come capaci di causare mutazioni ereditarie nelle cellule germinali umane.
Categoria 1B:	La classificazione nella categoria 1B si basa su: — risultati positivi di test in vivo di mutagenicità su cellule germinali di mammiferi, o — risultati positivi di test in vivo di mutagenicità su cellule somatiche di mammiferi, associati a dati che dimostrano che la sostanza può causare mutazioni nelle cellule germinali. Questi dati supplementari possono provenire da test in vivo di mutagenicità/genotossicità su cellule germinali o dimostrare la capacità della sostanza o dei suoi metaboliti di interagire con il materiale genetico delle cellule germinali, o — risultati positivi di test che dimostrano effetti mutageni in cellule germinali umane, ma non la trasmissione delle mutazioni alla progenie; per esempio, un aumento della frequenza dell'aneuploidia negli spermatozoi dei soggetti esposti.
CATEGORIA 2:	Sostanze che destano preoccupazione per il fatto che potrebbero causare mutazioni ereditarie nelle cellule germinali umane La classificazione nella categoria 2 si basa su: — risultati positivi di esperimenti su mammiferi e/o in taluni casi di esperimenti *in vitro*, ottenuti per mezzo di: — test in vivo di mutagenicità su cellule somatiche di mammiferi, o — altri test in vivo di genotossicità su cellule somatiche confermati da risultati positivi di test *in vitro* di mutagenicità. Nota: Le sostanze che danno risultati positivi in test *in vitro* di mutagenicità su mammiferi e che presentano un'analogia nella relazione struttura chimica-attività con sostanze di cui è accertata la mutagenicità sulle cellule germinali sono prese in considerazione per la classificazione come sostanze mutagene di categoria 2.

Una valutazione della sicurezza delle sostanze, in particolare di quelle classificate come sostanze CMR 1A o 1B, dovrebbe tenere conto dell'esposizione complessiva a tali sostanze provenienti da tutte le fonti. Di conseguenza, è opportuno che la Commissione, in stretta cooperazione con il CSSC, l'Agenzia europea per le sostanze chimiche (ECHA), l'Autorità europea per la sicurezza alimentare (EFSA) e altre parti interessate, proceda con urgenza a una revisione e metta a punto linee guida riguardanti la produzione e l'utilizzo delle stime relative all'esposizione complessiva a tali sostanze.

La valutazione da parte del CSSC concernente l'impiego di sostanze classificate come CMR 1A e 1B nei prodotti cosmetici dovrebbe tener conto anche dell'esposizione a tali sostanze dei gruppi di popolazione vulnerabili, come bambini di età inferiore a tre anni, persone anziane, donne incinte e che allattano nonché persone con un sistema immunitario compromesso

Sperimentazione animale

La sperimentazione animale va sostituita con metodi alternativi. Il regolamento vieta la realizzazione di sperimentazioni animali all'interno dell'Unione europea per: i prodotti finiti; gli ingredienti o le combinazioni di ingredienti.

Il regolamento vieta altresì l'immissione sul mercato europeo di prodotti la cui formulazione finale sia stata oggetto di una sperimentazione animale; prodotti contenenti ingredienti o combinazioni di ingredienti che siano stati oggetto di una sperimentazione animale.

La direttiva 86/609/CEE del Consiglio, del 24 novembre 1986,
concernente il ravvicinamento delle disposizioni legislative, regolamentari e amministrative degli Stati membri relative alla protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici(1)
GU L 358 del 18.12.1986, pag. 1. (1) ha stabilito regole comuni per l'utilizzo degli animali a fini sperimentali nella Comunità e ha fissato le condizioni alle quali tali esperimenti devono essere condotti nel territorio degli Stati membri. In particolare, l'articolo 7 di tale direttiva prescrive che gli esperimenti su animali siano sostituiti da metodi alternativi, laddove essi esistano e siano scientificamente validi.

(40) È possibile garantire la sicurezza dei prodotti cosmetici e dei loro ingredienti attraverso metodi alternativi non necessariamente applicabili a tutti gli usi dei componenti chimici.

La sicurezza degli ingredienti utilizzati nei prodotti cosmetici potrà essere garantita progressivamente applicando metodi alternativi che non comportino l'impiego di animali, convalidati a livello comunitario, oppure approvati in quanto scientificamente validi dal Centro europeo per la convalida di metodi alternativi (ECVAM) e tenendo nel debito conto lo sviluppo della convalida in seno all'Organizzazione per la cooperazione e lo sviluppo economici (OCSE).

REPLACEMENT **sostituzione degli animali con tecniche alternative**

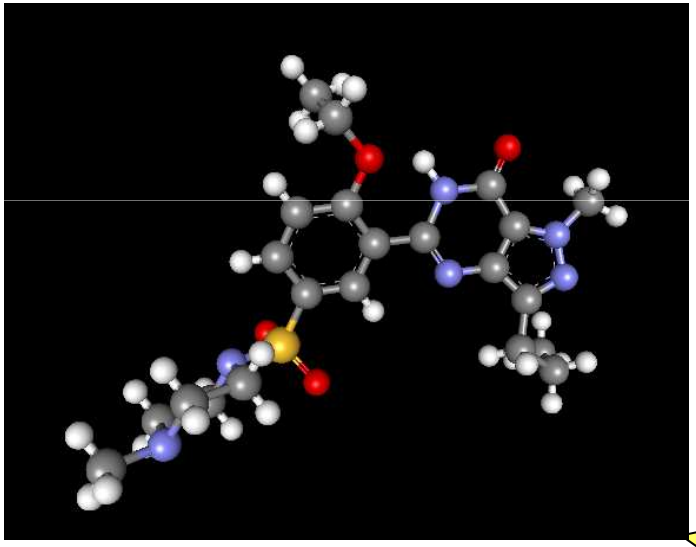
REFINEMENT **ottimizzazione dei protocolli sperimentali per minimizzare la sofferenza degli animali.**

REDUCTION **riduzione del numero degli animali mediante la conduzione di test multipli sugli stessi**

Computational Toxicology

Metodi computazionali (in silico) possono essere utilizzati per predire gli effetti delle sostanze chimiche sulla salute dell'uomo e delle specie animali ed il loro destino ambientale

Proprietà chimico-fisiche



Tossicità

Animali e costi dei test

Destino ambientale

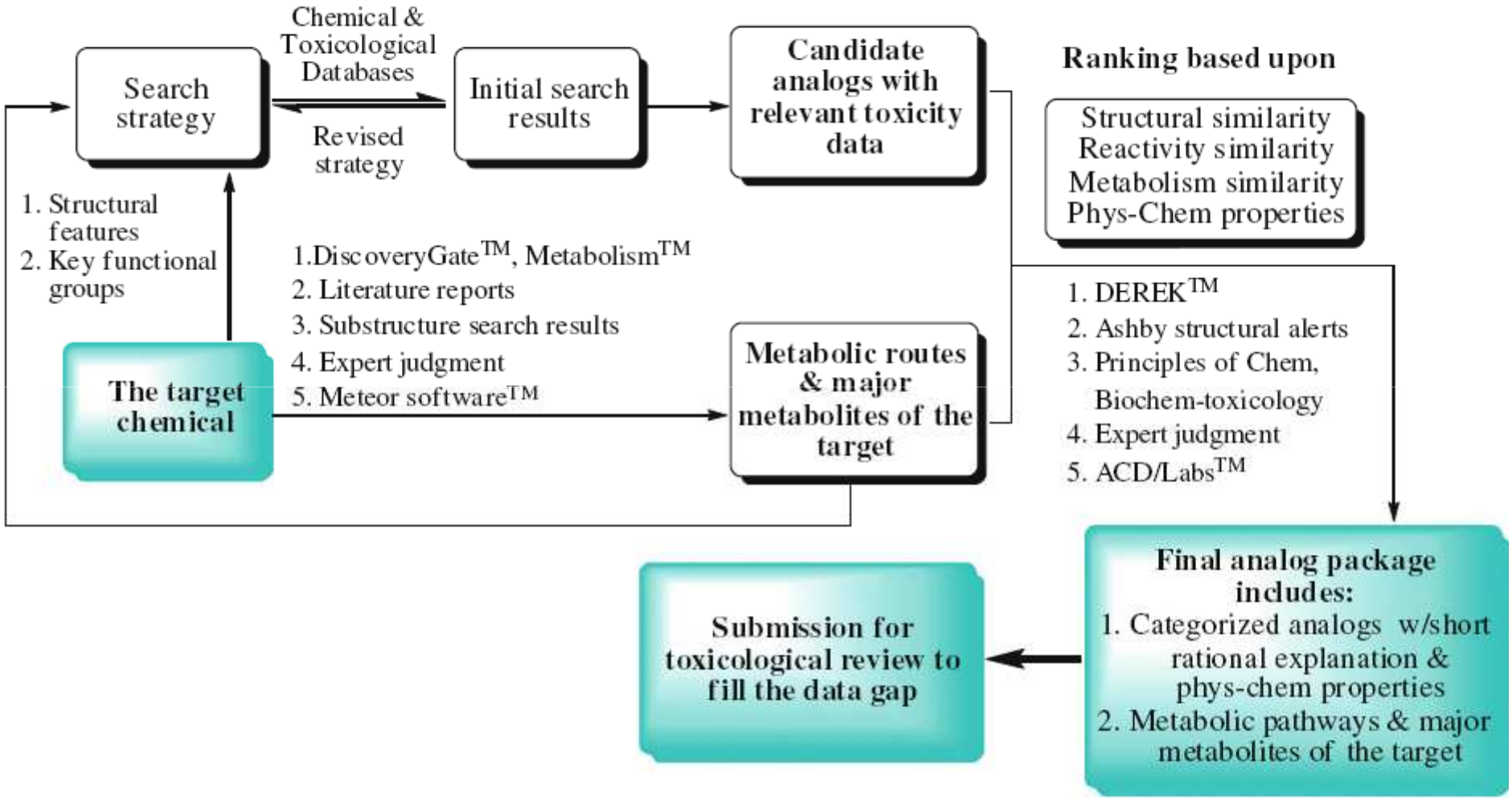
Assorbimento, Distribuzione, Metabolismo. Eliminazione (ADME)

(Q)SAR

The REACH regulation foresee the application of (Q)SARs in a number of ways, mainly to:

- **provide information for use in priority setting procedures**
- **guide the design of an experimental test or testing strategy**
- **improve the evaluation of existing test data**
- **provide mechanistic information (which could be used, for example, to support the grouping of chemicals into categories)**
- **fill data gaps for classification and labelling and for risk assessment.**

READ ACROSS


Chemical & Toxicological Databases
Search strategy
Revised strategy
Initial search results
Candidate analogs with relevant toxicity data
Ranking based upon
Structural similarity
Reactivity similarity
Metabolism similarity
Phys-Chem properties
1. Structural features
2. Key functional groups
1.DiscoveryGate™, Metabolism™
2. Literature reports
3. Substructure search results
4. Expert judgment
5. Meteor software™
The target chemical
Metabolic routes & major metabolites of the target
1. DEREK™
2. Ashby structural alerts
3. Principles of Chem, Biochem-toxicology
4. Expert judgment
5. ACD/Labs™
Final analog package includes:
1. Categorized analogs w/short rational explanation & phys-chem properties
2. Metabolic pathways & major metabolites of the target
Submission for toxicological review to fill the data gap

Metodi Alternativi: test in vitro

I sistemi in vitro permettono l'identificazione di metaboliti tossici in ambiente controllato e sono importanti per la definizione del meccanismo d'azione dei composti

Genotossicità (Luglio 2010)
Test del micronucleo in vitro

Irritazione dermica (Luglio 2010)
Metodi in vitro sull'epidermide umana ricostituita

Sensibilizzazione cutanea (Luglio 2010)
OECD Test Guidelines No. 429 Local Lymph Node Assay (LLNA)

test di trasformazione cellulare in vitro (CTA) predittivo della cancerogenesi (marzo 2012)