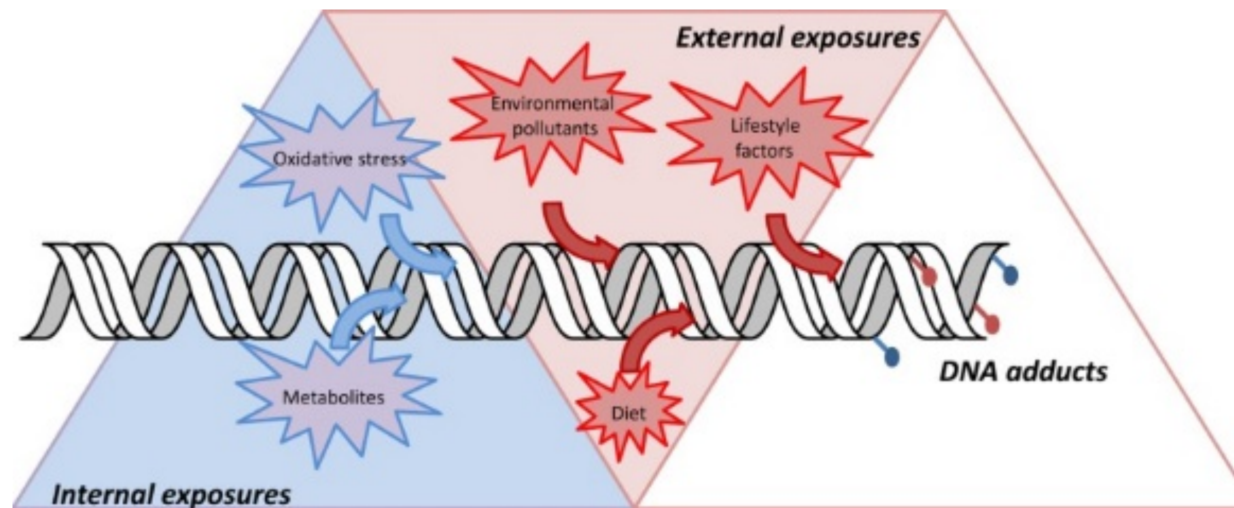
Methods in Genetic Toxicology

An Overview by Yves Haufe

Agenda

- Introduction
- Methods in genetic toxicology I – endpoint mutations
- Methods in genetic toxicology II – indicator tests
- Methods in genetic toxicology III – animal experiment



Balbo S, Turesky R, Villalta P. *DNA adductomics*. **Chemical research in toxicology, 2014**; 27(3): **356–366**

Introduction I - Definitions

Targets in TOXICOLOGY Research Training Group

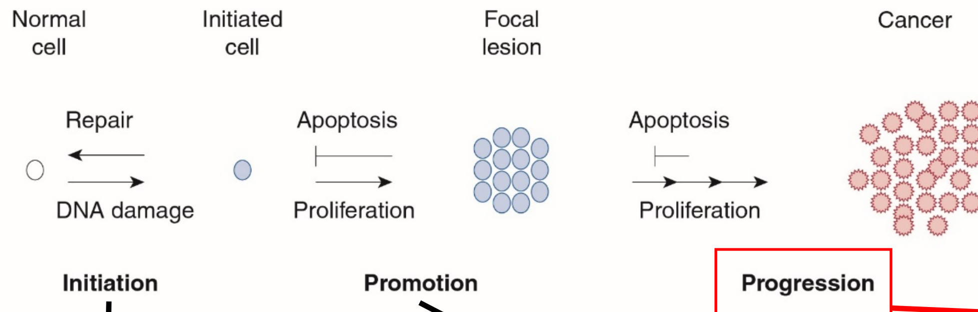
- **Cancerogenicity:** ability of a carcinogen to lead to a statistically increased number in neoplasms (compared to control)
- **Genotoxicity:** the ability of a factor to damage the integrity of the DNA regardless of the mechanism
  - Directly: DNA-Adducts, strand breaks, Crosslinks
  - Indirectly: spindle apparatus, increase in error rate of DNA-Polymerases
- **Non-Genotoxicity** = epigenetic alterations (cytotoxicity, receptor-mediated, hormonal, methylation, ROS)
- **Mutagenicity:** ability of a factor to permanently alternate the DNA

Causal order: genotoxic event →→ mutation →→→ cancer

Klaassen, CD (Ed.). (2013). ***Casarett & Doull's Toxicology***. *The basic science od poisons* (8th ed.). McGraw-Hill Education

Introduction II - Carcinogenesis

Principle of Multistage Model



Genotoxic agents

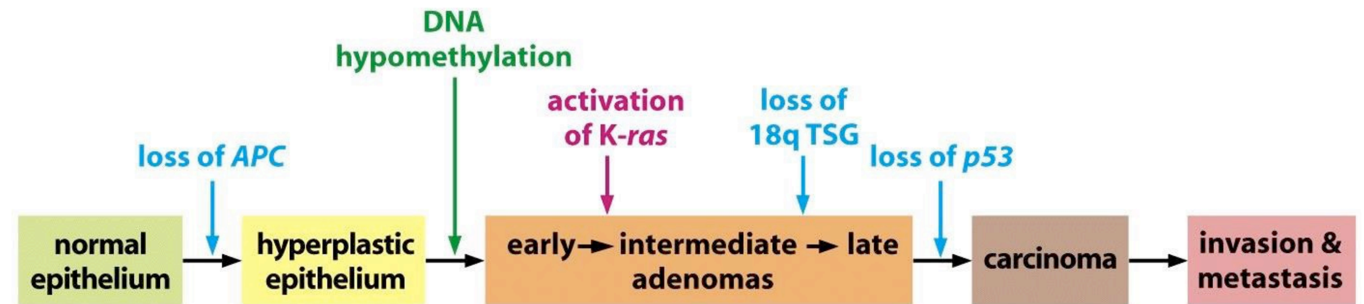
- One cell division needed to fixate
- mutations aren't reversible
- Alone → no cancer
- Single exposure may be sufficient
- No Threshold!

Non-Genotoxic agents

- multiple cell division needed
- reversible
- Alone → no cancer
- prolonged exposure necessary
- Threshold!

Complete carcinogens act on both stages

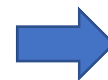
Colon Cancer



Accumulation of several more mutations

Determination of genotoxicity is not the measurement of the appearance of cancer but:

- It is a causal, initiative event in carcinogenesis
- proven association between positive tests for genotoxicity and mutagenicity in human and animals



Any genotoxicant will be carcinogenic unless proven otherwise

Klaassen, CD (Ed.). (2013). ***Casarett & Doull's Toxicology**. The basic science od poisons* (8th ed.). McGraw-Hill Education

Introduction III – important considerations

Targets in TOXICOLOGY Research Training Group

Prokaryotic vs eukaryotic cells

Ames test cannot be a stand alone assay

In-vitro vs *in-vivo* (**Bioactivation!**)

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A lot of agents with carcinogenic potential needs to be activated (Phase I reaction)

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In-vivo biotransformation system is complex and specie dependent (polymorphism)

Endogenous or heterologous enzymes of cell

External biotransformation:

- *Ex-vivo* tests
- Host-mediated assay
- Purified enzymes
- **S9-Mix**
  (Liver homogenate of Arochlor 1254 treated rats + NADPH system)

Somatic vs germline cells

- **Germline:** higher impact on species because hereditable
- **Somatic:** cancer of the individual

Methods I – Tests for mutations

Targets in TOXICOLOGY Research Training Group

Endpoint: Mutations

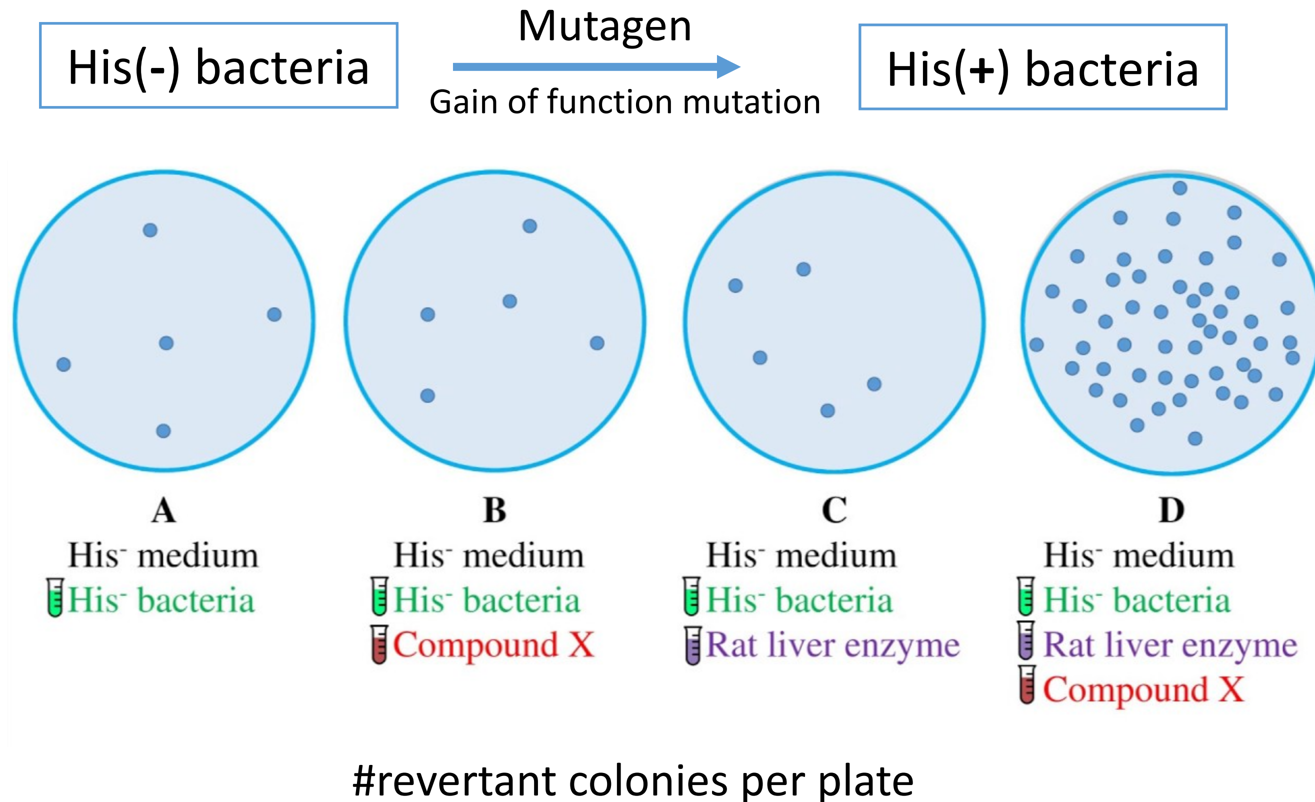
→ change in phenotype is used as surrogate for mutation
→ direct visualisation of chromosomes

Gene mutations	Structural mutations (clastogens)	Numeric mutations (aneugens)
Ames test (OECD 471)	Chromosome abberation assay (OECD 473)	
HPRT-test (OECD 476)	Chromosome painting (FISH based)	
TK-Test / MLA (big colonies) (OECD 490)	**Micronukleus test (OECD 487, 474)**	
Pig A-Assay	Sister chromatid exchanges (SCE Test)	
whole genome sequencing	TK Test / MLA (small colonies) (OECD 490)	
Cleaved amplified polymorphic sequence (CAPS)		

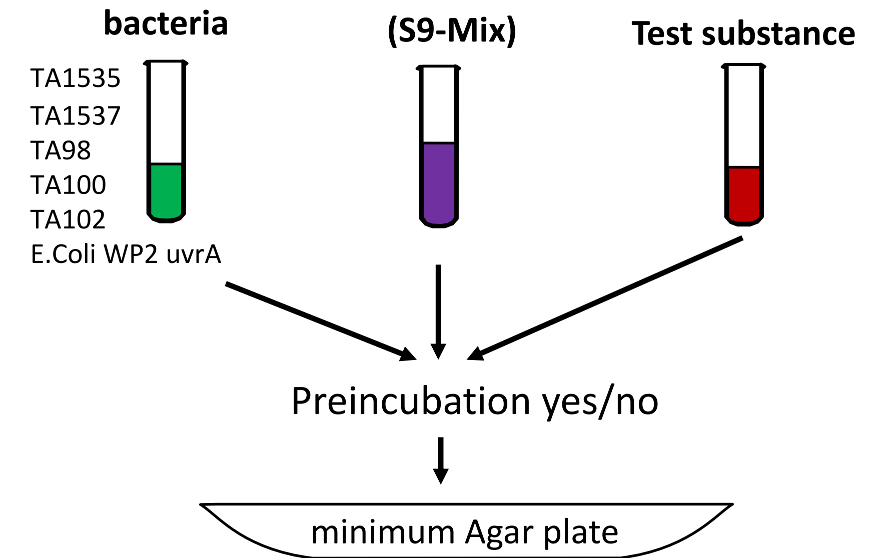
➡ These methods show mutagenic potential as a result of a genotoxic action

HPRT – hypoxanthin phosphoribosyl transferase, TK – Thymidine Kinase, MLA – Mouse lymphoma assay

Methods I – Ames Test



#revertant colonies per plate

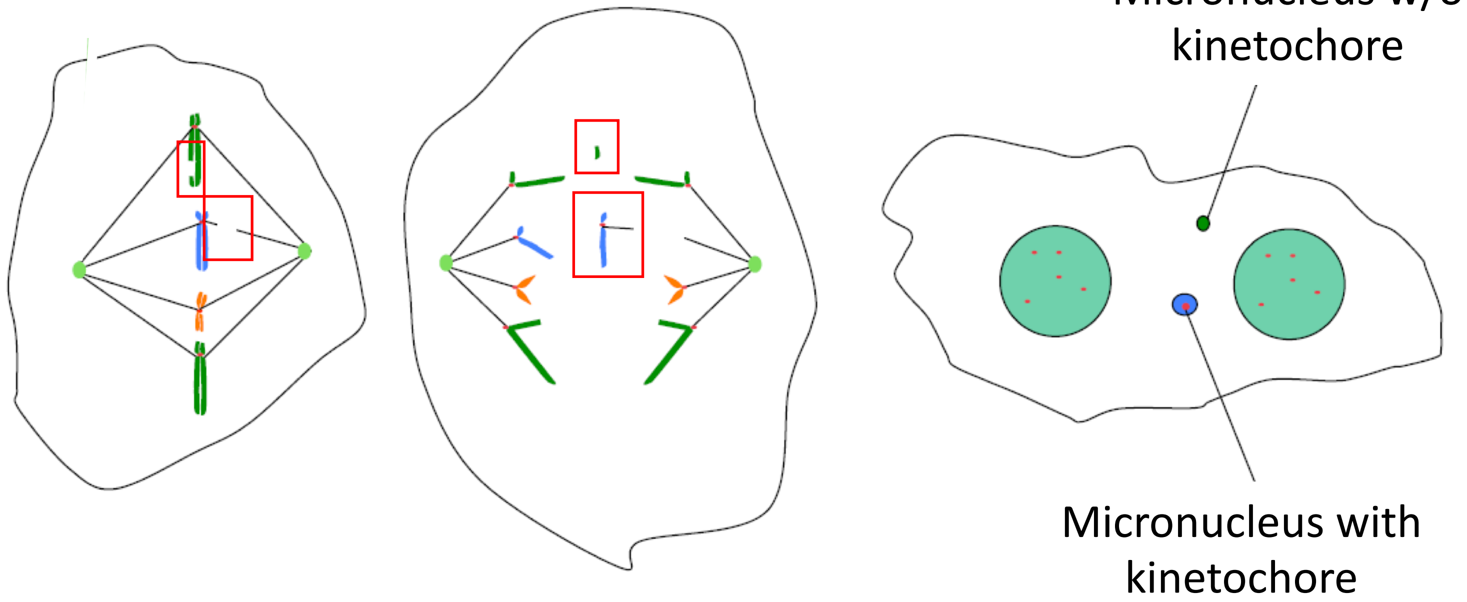


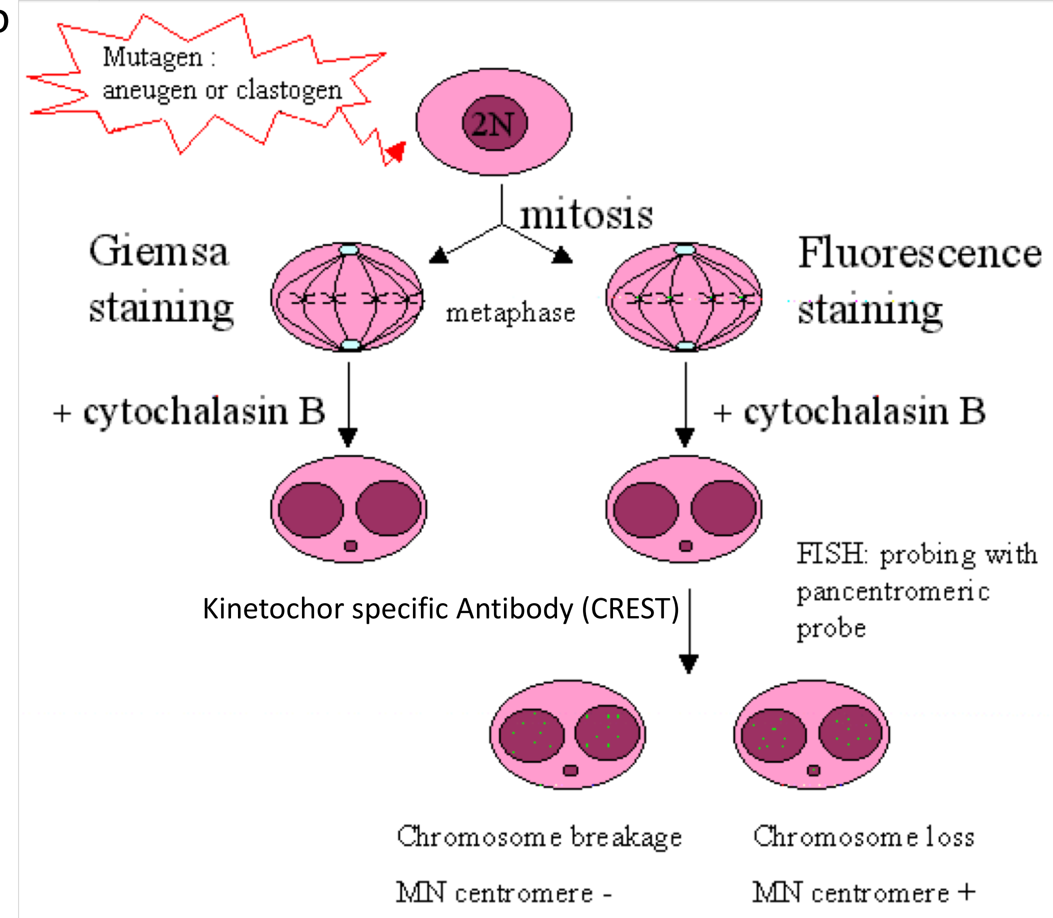
- Routine screening assay (validated)
- Easy to perform and robust
- Positive results shows already MoA

- Not suitable for bactericidal agents
- Detects only gene mutations
- **Negative results** need to be confirmed

similar principle for TK- and HPRT-Test but loss of function
(see additional information)

Methods I – Micronuclei Test





genotoxic events detected by classical micronucleus assay:

- Acentric chromosome fragments
- Modified chromosome structures
- Segregation errors

Klaassen, CD (Ed.). (2013). ***Casarett & Doull's Toxicology***. *The basic science od poisons* (8th ed.). McGraw-Hill Education

Methods I – Micronuclei Test

Targets in TOXICOLOGY Research Training Group

- Analysis traditionally by hand
  - 2000 cells (#MN/1000 cells)
  - Frequency of MN
  - Mitotic potential

- Using of FACS to automate analysis
  - Double staining to detect apoptotic cells
  - Latex beats as standard to detect vital cells

- *in-vitro* Assay (OECD 487)
- *In-vivo* assay (OECD 474) with immature erythrocytes in the bone narrow of treated mice (or peripheral blood)

- Best assay to test for aneugenic potential (no artefacts)
- Standardized and validated (can be automated)
- Easily expandable (e.g. cytosin arabinoside to block base excision repair)

- Requires cell division or stable Micronucleus

Fenech M. *The in vitro micronucleus technique*. **Mutation research, 2000**; 455(1-2): **81–95**

Methods II – Indicator tests

Targets in TOXICOLOGY Research Training Group

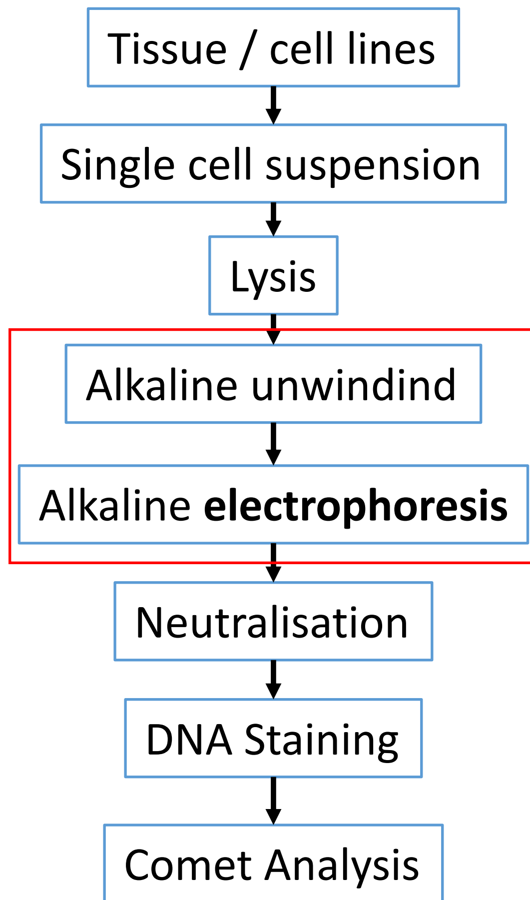
Endpoint: genotoxic event

→ DNA strand-breaks
→ Abasic sites (AP-sites)
→ DNA-DNA or DNA-Protein crosslinks
→ DNA-Adducts
→ Dimers
→ Oxidative damage
→ …

Prominent examples:

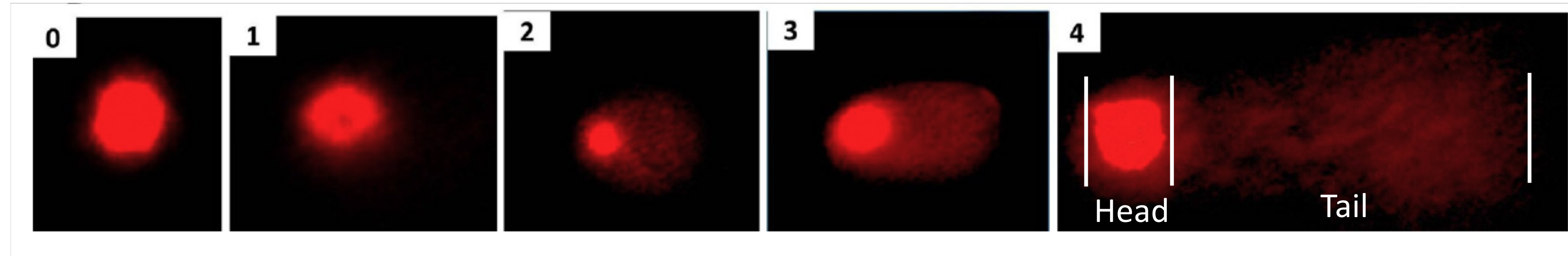
- Single cell gel electrophoresis - **Comet Assay**
- Transgenic mouse mutation assay
- Liver unscheduled DNA synthesis (UDS) Assay
- Assays for DNA-Adducts (**Pre/Post labelling**, LC-MS/MS, immune detection methods)

Methods II – (alkaline) Comet Assay



(OECD 489)

- ss/ds-breaks
- AP sites
- DNA cross-linking
- Incomplete excisison repair



100 nucleoids per slide, scoring either by eye (categories) or automated (intensity head vs tail)

- Applicability to various cell types
- Sensitivity for DNA damage
- Relative fast and low costs
- Professional software for automated analysis

- No differentiation cytotoxicity vs genotoxicity
- Unknown mechanism

Araldi R et al. *Using the comet and micronucleus assays for genotoxicity studies*. **Biomedicine & pharmacotherapy**, **2015**; 72: **74–82**

Methods II – DNA-Adducts

Radio-labelling methods

Pre-labelling

Treatment with radio-labelled (^{14}C) substance of interest, isolation/purification of DNA, measurement of decay

- Location of the labelling in the molecule
  - Which fragment binds to the DANN (false negative)
  - Do labeled fragments go into the host metabolism? → de-novo synthesis of DNA (false positive)
- Instable adducts (false-negative)

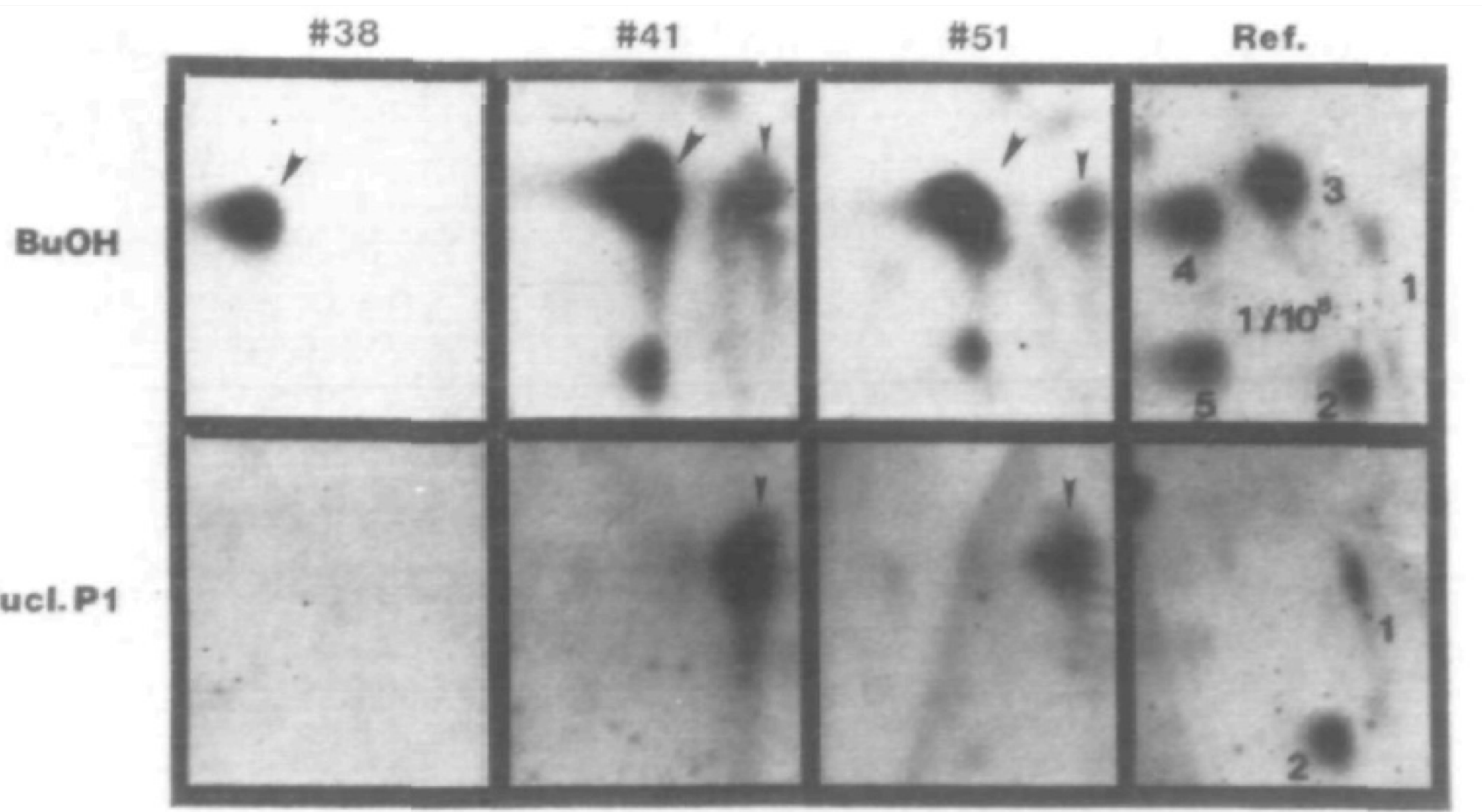
obsolete

Post-labelling

- **Digestion of isolated DNA** → 3‘-desoxynucleoside monophosphates
- **[Accumulation of adducts** (increase sensitivity)**]**
  - 1-Butanol extraction (highly lipophilic adducts)
  - Nuklease P1 (unadducted nucleosides are faster degraded)
  - Solid phase extraction
- **T4 poly-nucleotide kinase**: transfer of [γ-^{32}P]ATP to 5’-OH
- **Separation and detection**
  - Multidirectional thin layer chromatography (autoradiography) and quantification by scintillation counting
  - HPLC with on-line radio-detector

Methods II – 32P Post-Labelling

DNA from oral mucosa of Indian tobacco chewers



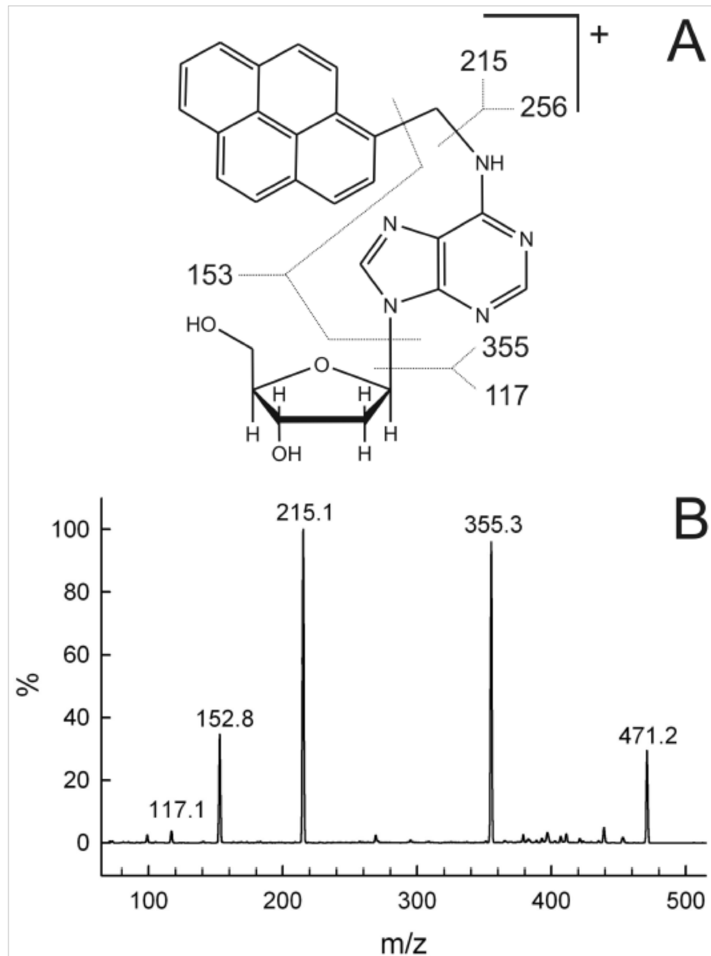
Beach A und Gupta R. *Human biomonitoring and the 32 P-postlabeling assay*. **Carcinogenesis**, **1992**; 13(7): **1053–1074**

- Small amount if sample (1-5 µg of DNA)
- High sensitivity 1 in 10^8 - 10^{10} nucleotides

- Use of radioactive isotops
- Influence of accumulation step on adducts‘ unknown
- Small adducts hard to detect
- No internal standards!
  → losses in preparation or incomplete labelling
- **Lack of structural information**
- Low through-put

Methods II – MS techniques

1-Methylpyren-Adenosin adduct and its fragmentation



- Exact structure
- Precise quantification
- High through-put

- Expensive in equipment and analysis
- Larger sample amount (10 - 100µg)
- Lower sensitivity
- **Difficult to perform for unknown adducts**

Combination of Post-Labelling and MS-Analysis in the beginning

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chip-based technologies for online column switching which combine online sample cleanup, capillary separation, and nanoESI (1 adduct in 10^9 nucleotides using 1–10 µg of DNA)

↓

Continous improvements (e.g. LC-MS/MS Adductomics) will make Postlabeling obsolete in near future

Villalta P und Balbo S. *The Future of DNA Adductomic Analysis*. **International Journal of Molecular Sciences**, **2017**; 18(9): **1870**

Klaene J, Sharma V, Glick J, Vouros P. *The analysis of DNA adducts*. **Cancer letters**, **2013**; 334(1): **10–19**

Methods III – Carcinogenicity testing

Only provable in animal experiments:
- OECD 451 for chemicals
- ICH S1B for pharmaceuticals

- **Species**: 2 species (rat, mice, hamster)
- **Duration**: Life-Time (around 18 – 24 months)
- **Application**: relevant human exposure route
- **Dose level**: mostly 3 (¼ MTD, ½ MTD, MTD)
- **Animal numbers**: 50 – 60 animals /group/sex
- **Costs**: ca. 2 Mio Dollars

MTD – maximal tolerated dose

Statistical aspects:

Lowest effect to be detected with statistical significance in animal experiment:
- 0 neoplasms in control group
- 4 in treated group

→ Increase of 8% within 50 animals

↓

Roughly 8.000 – 80.000 animals with induced cancer in 10^6 exposed animals
Less than 1 case of induced cancer in 10^6 exposed human acceptable

↓

Given a linear dose-response curve, dosage in animal exposure should exceed human one about a factor of 8.000 – 80.000

➡ Principle of the Margin of Exposure (MoE)

Take Home Message

- Presumption of any regulatory agency: Any genotoxic/mutagenic action is carcinogenic unless proven otherwise
- Genotoxic testing always includes a whole battery of test:
  - Ames Test (mostly for screening)
  - In vitro mammalian cell gene mutation test (TK/HPRT tests)
  - *in-vitro/in-vivo* chromosome mutation test (Micronucleus)
  - Suitable Indicator test (Comet-Assay, UDS-Test, DNA-Adducts, SOS-Chromotest, umu-test, …)
- Negative results in each assay needs to be further discussed/investigated

Thank you for your attention!

Any Questions?

Additional information – TK/HPRT test

Targets in TOXICOLOGY Research Training Group

TK test

- TK+/- in L5178Y mouse or TK6 human lymphoma cells (autosomal gene)
- Cultivation in suspension / semi-solid agar
- Selection: Trifluorothymidine

- Able to distinguish gene and structural chromosome mutations
  - Gene mutation: bigger colonies
  - Str. Chromosome mutations: smaller colonies

- "**Loss of function**" – more sensitive (Methotrexate inhibits de-novo synthesis of nucleotides, few spontaneous mutants)

HPRT test

- HPRT in V79 or CHO (gonosomal gene)
- Cultivation on plates like Ames test
- Selection: 6-Thioguanine (Mismatch repair dead-end)

- 1000 times bigger genome compared to bacteria
- More complicated culture
- Heterozygous or gonosomal genes
- Proliferation in colonies necessary (longer generation time)