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Chemical reactivity of nucleic acids
Chemical methods in DNA studies
DNA damage

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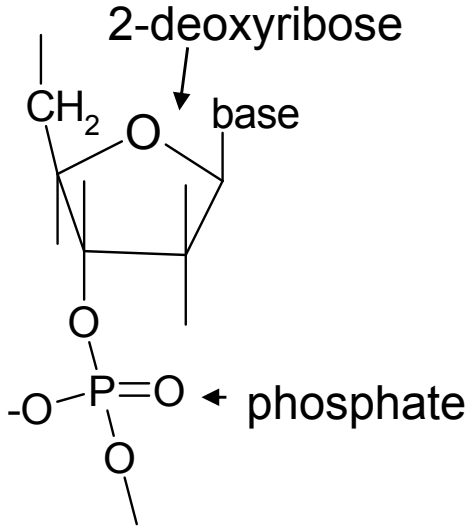
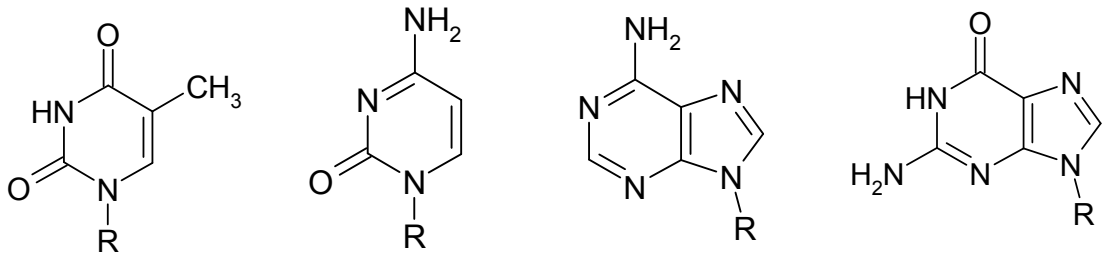
Chemical reactivity of DNA

- DNA chemistry is derived from chemistry of its costituents
- phosphodiester bonds
- N-glycosidic bonds
- deoxyribose
- nitrogenous bases



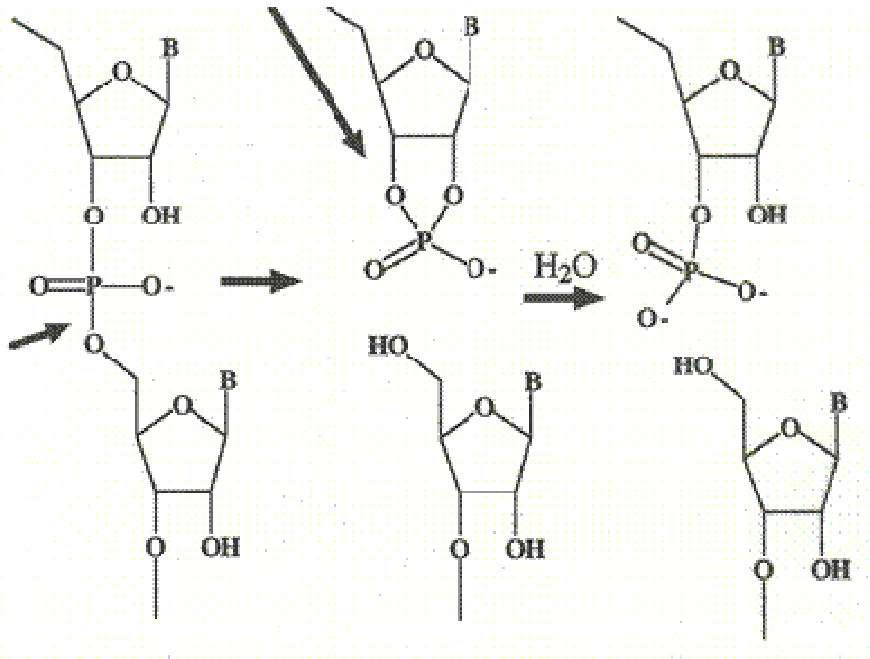


Chemical modification of DNA:

- damage to the genetic material
- analytical use

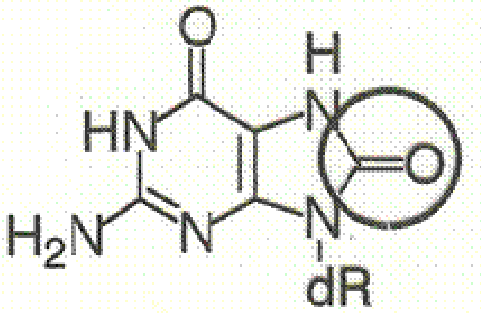
DNA hydrolysis

- both phosphodiester and N-glycosidic bonds susceptible to acid hydrolysis
- N-glycosidic bond more stable toward hydrolysis in pyrimidine than in purine nucleosides (and more in ribo- than in deoxynucleosides)
- stable in alkali (unlike RNA)
- alkali-labile sites: upon DNA d
- enzymatic hydrolysis (N-glycc phosphodiesterases)

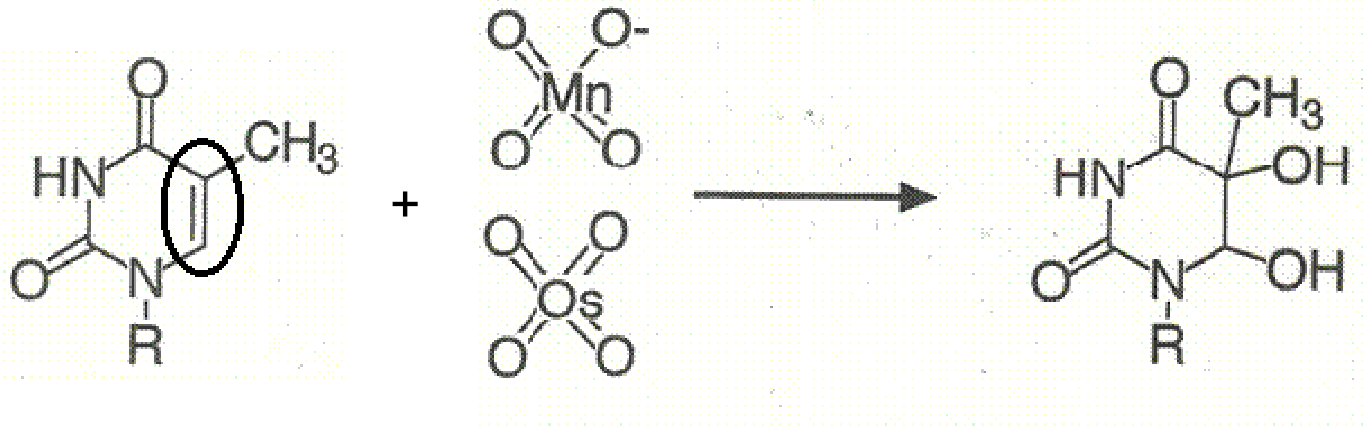


Oxidation

- two main sites susceptible to oxidation attacks:
  - C8 of purines (ROS)

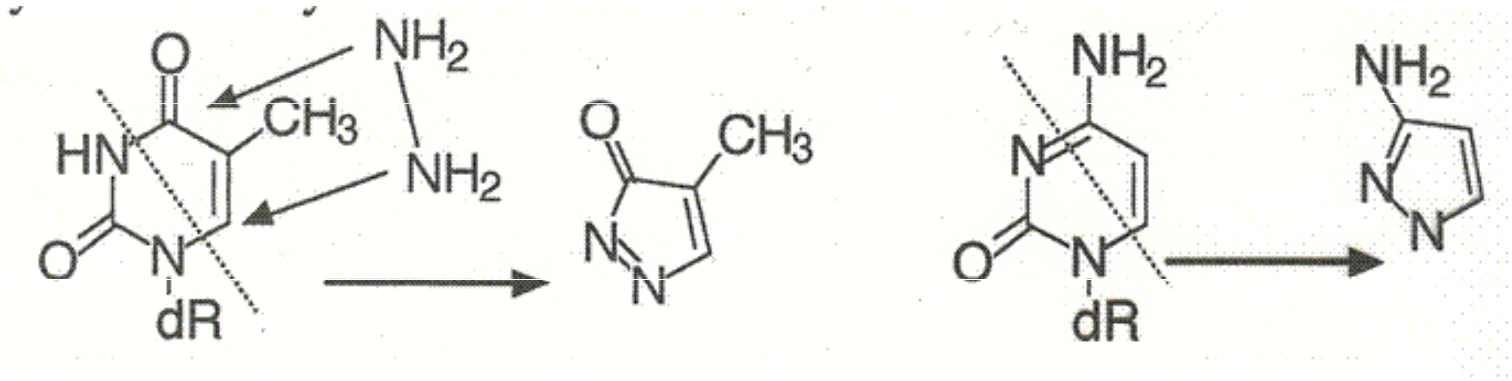


  - C5-C6 of pyrimidines



reactions with nucleophiles

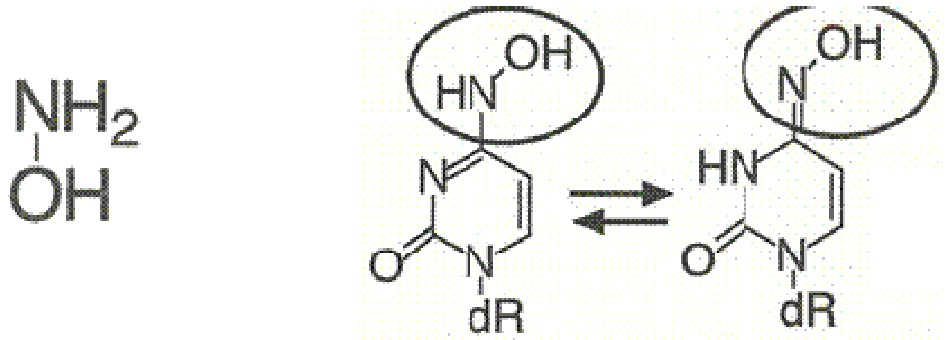
- C4 and C6 are centres of electron deficit in pyrimidine moieties



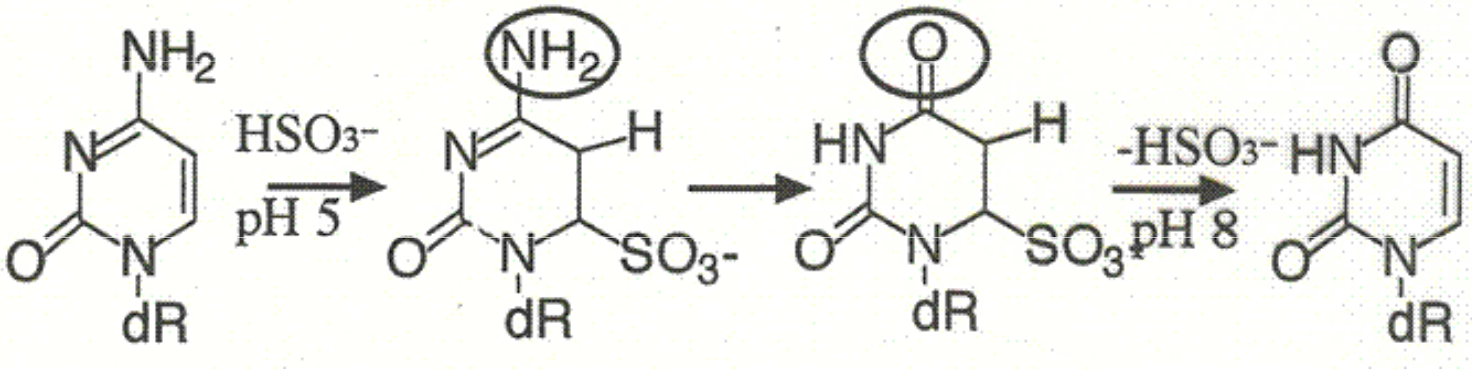
- reaction with hydrazine: pyrazole derivative and urea residue bound to the sugar
- with T the reaction is disfavored in high salt: Maxam-Gilbert sequencing technique

reactions with nucleophiles

- hydroxylamine: cytosine modification
- the products‘ preferred tautomer pairs with adenine →mutagenic

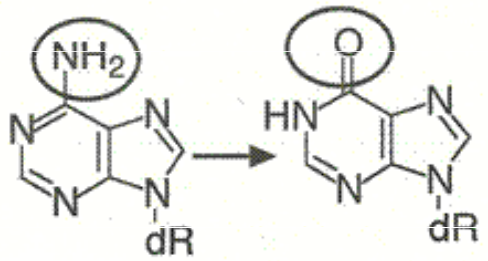


- bisulphite: cytosine modification inducing its deamination to uracil →mutagenic
- 5-methyl cytosine does not give this reaction: genomic sequencing of $5^{m}C$

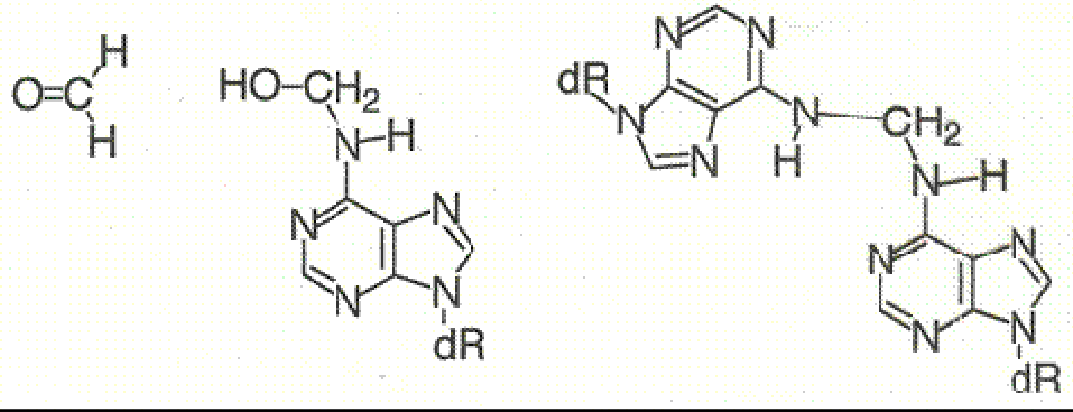


reactions with electrophiles

- attacking N and/or O atoms
- nitrous acid (HNO_2) causes base deamination (C→U, A→I) – affecting base pairing, mutagenic

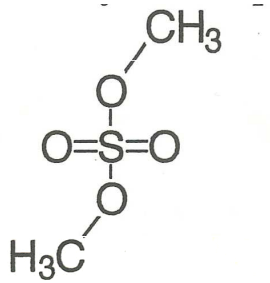


- aldehydes: reactions with primary amino groups
- formaldehyde: two step reaction

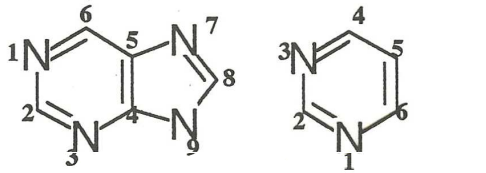


DNA alkylation

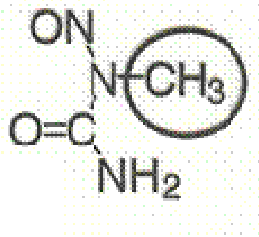
- hard or soft alkylating agents
- hard ones attack both N and O atoms, soft only N
- dimethyl sulfate: typical soft alkylating agent



G-N7 >A-N1>C-N3>T-N3



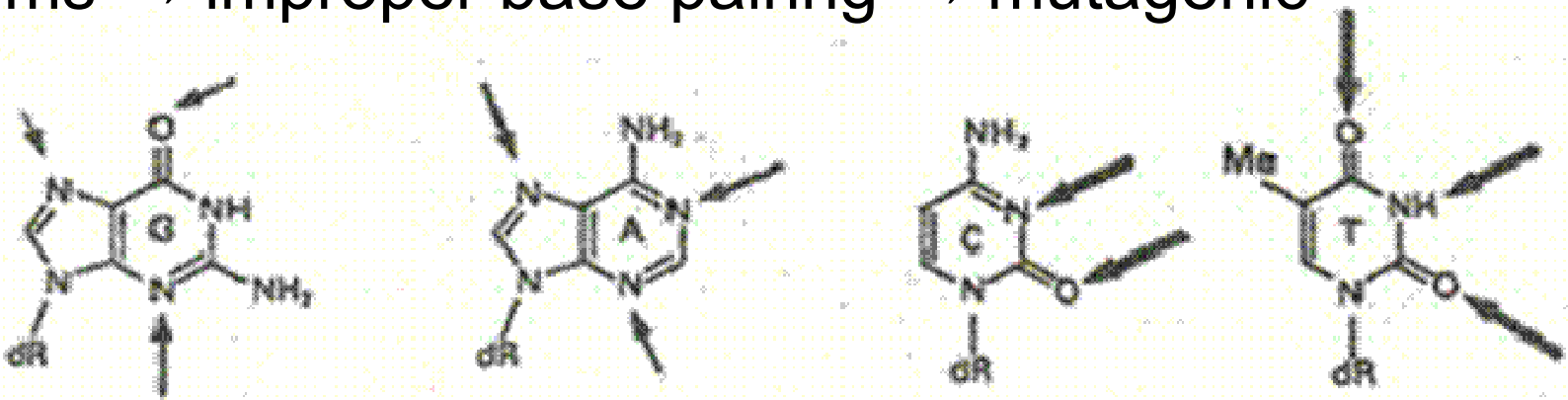
- N-alkyl-N-nitroso urea: typical hard alkylating agent
- modifies all N + O in bases as well as phosphate groups (forming phosphotriesters)
- analytical use (sequencing, foorprinting)



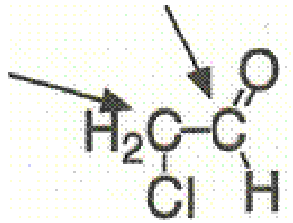
Biological consequences of base alkylation

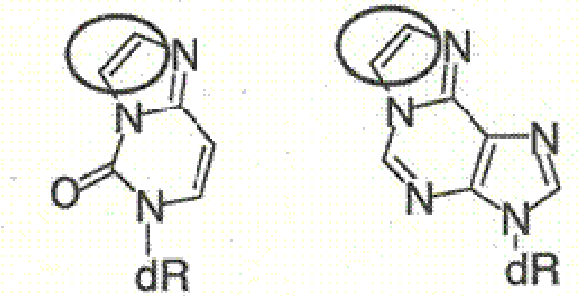
- **N-alkylation**: the primary site = N7 of guanine (accessible in both ss and dsDNA)
  - does not change base pairing; easily repairable
- N3 of adenine or guanine: located in minor groove
  - cytotoxic modification (DNA/RNA polymerization blocked)
- N1 of guanine: interferes with base pairing

- **O-alkylation (G-O6, T-O6)** the bases „locked“ in enol forms → improper base pairing → mutagenic

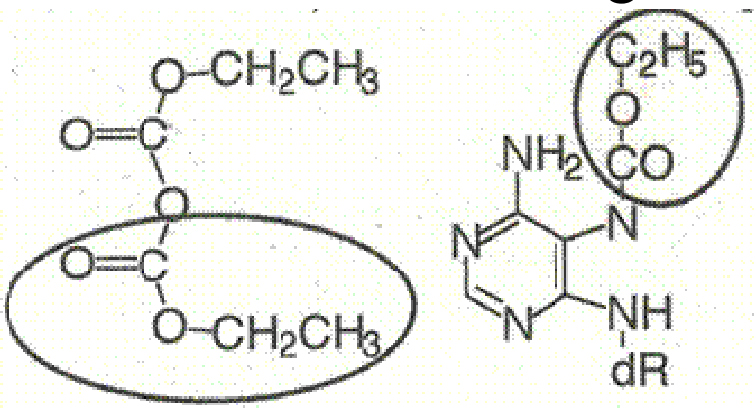


- **chloro- (bromo-) acetaldehyde**: two reactive centres (aldehyde and alkylhalogenide)
- reaction with C or A
- chemical probes (react only with unpaired bases)





- **diethyl pyrocarbonate**: acylation of purines (primarily A) at N7
- modification leads to opening of the imidazole ring
- chemical DNA probing

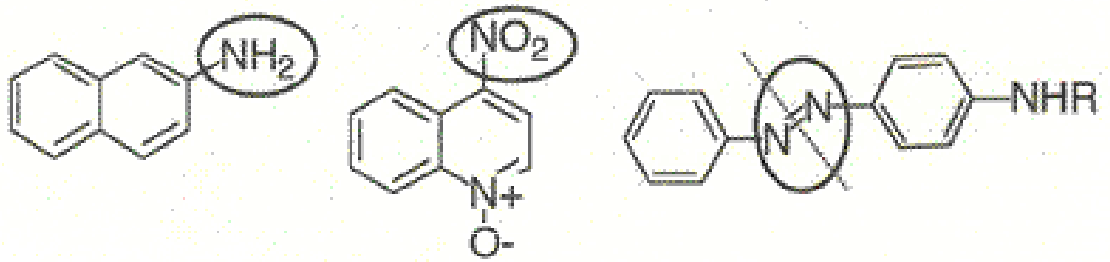


Metabolically activated carcinogens

- some substances became toxic after their metabolic conversion
- **detoxifying** machinery of the organism acts here as a bad fellow
- microsomal hydroxylase complex, **cytochrome P450**
- the role of this system is to introduce suitable reactive groups into xenobiotics enabling their conjugation with other molecules followed by removal from the organism
- **but….**

Metabolically activated carcinogens

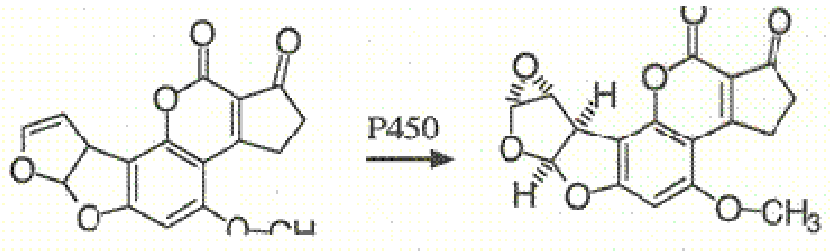
- aromatic nitrogenous compounds (amines, nitro- or azo- compounds):

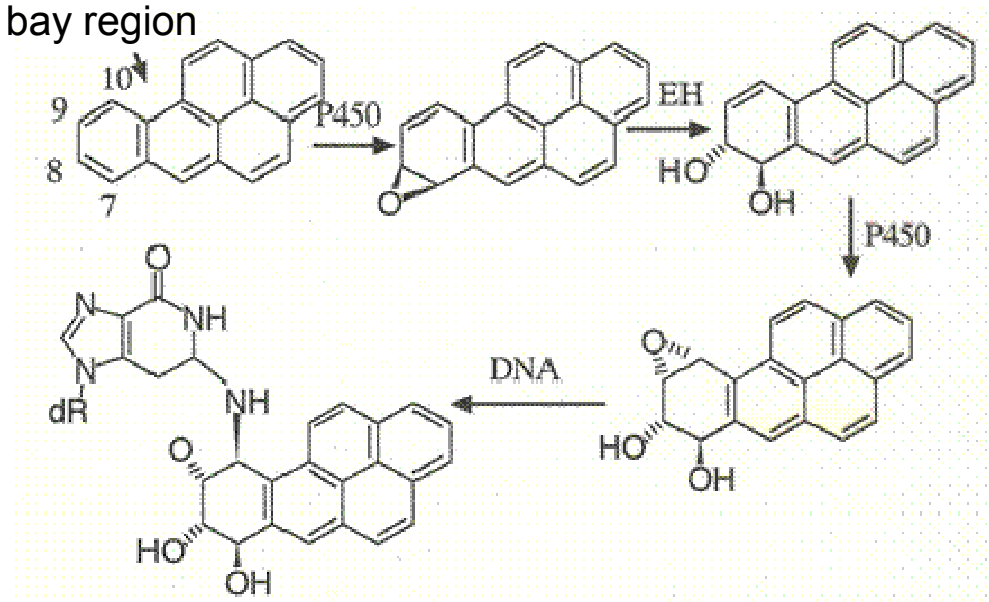


- aromatic amines are converted into either (safe) phenols, or (dangerous) hydroxylamine derivatives
- azo- compounds: „cleaved“ into amines
- nitro- compounds: reduced into hydroxylamines

Metabolically activated carcinogens

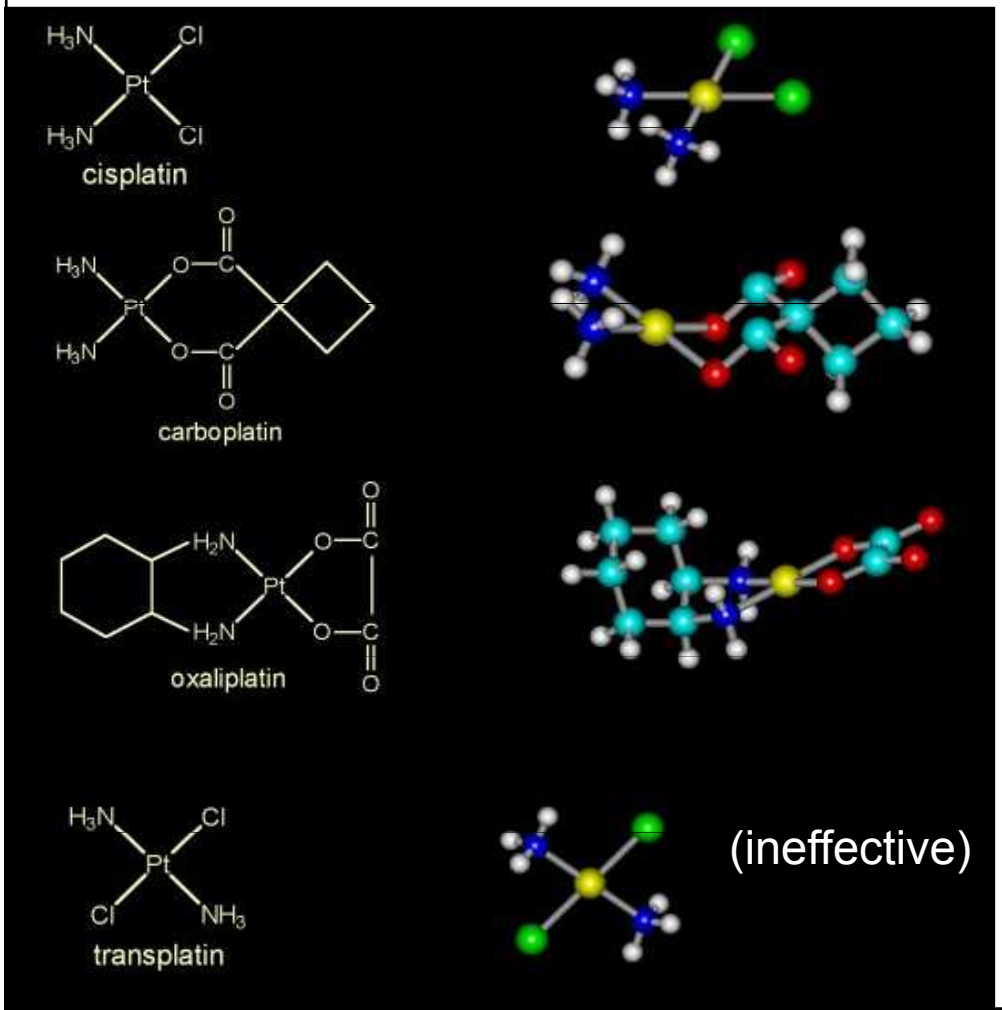
- polycyclic aromatic hydrocarbons like benzo[α]pyrene: three-step activation
  - P450 introduces epoxy group
  - epoxide hydrolase opens the epoxide circle
  - P450 introduces second epoxy group
- DNA adduct formation (primarily -NH_2 of guanine, then G-N7, G-O6 and A-N6)
- similar pathway of aflatoxin activation

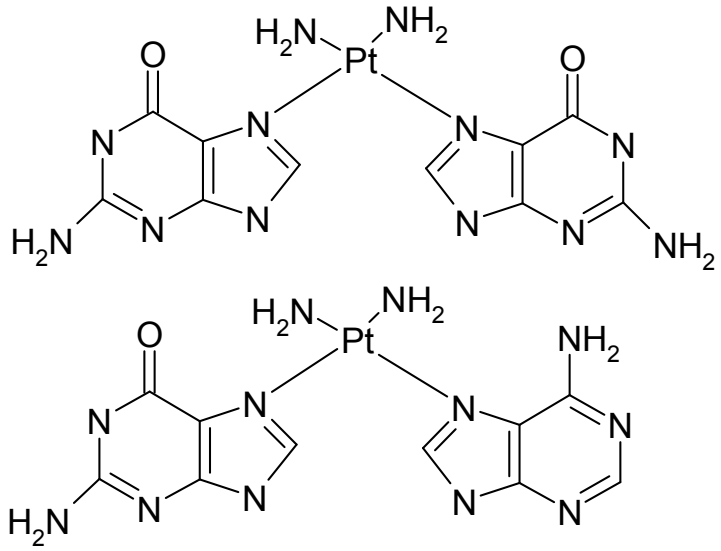




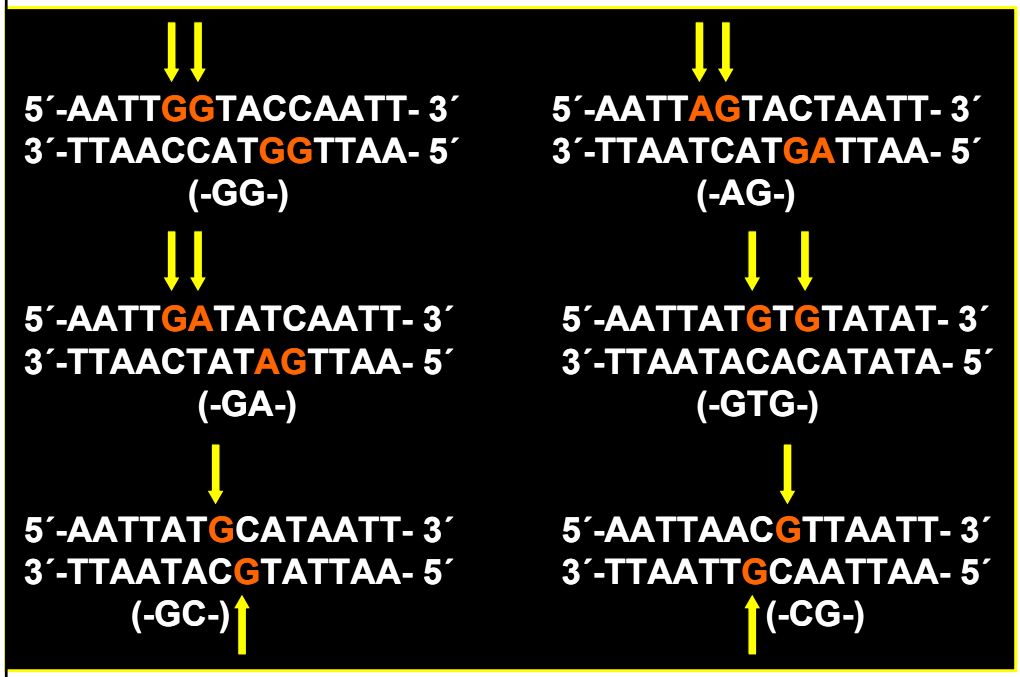
anticancer drugs

- some types of antineoplastic agents act via formation of DNA adducts
- metallodrugs: mainly platinum complexes



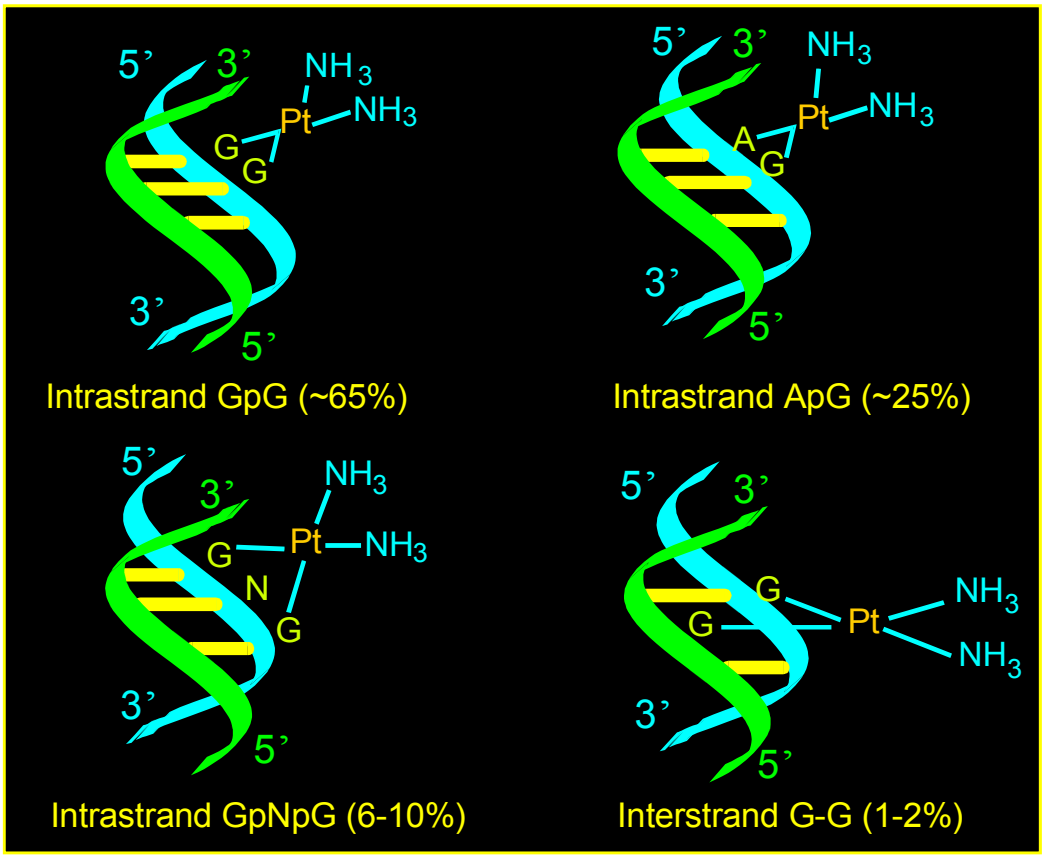


cisplatin: reaction with DNA in certain sequence motifs

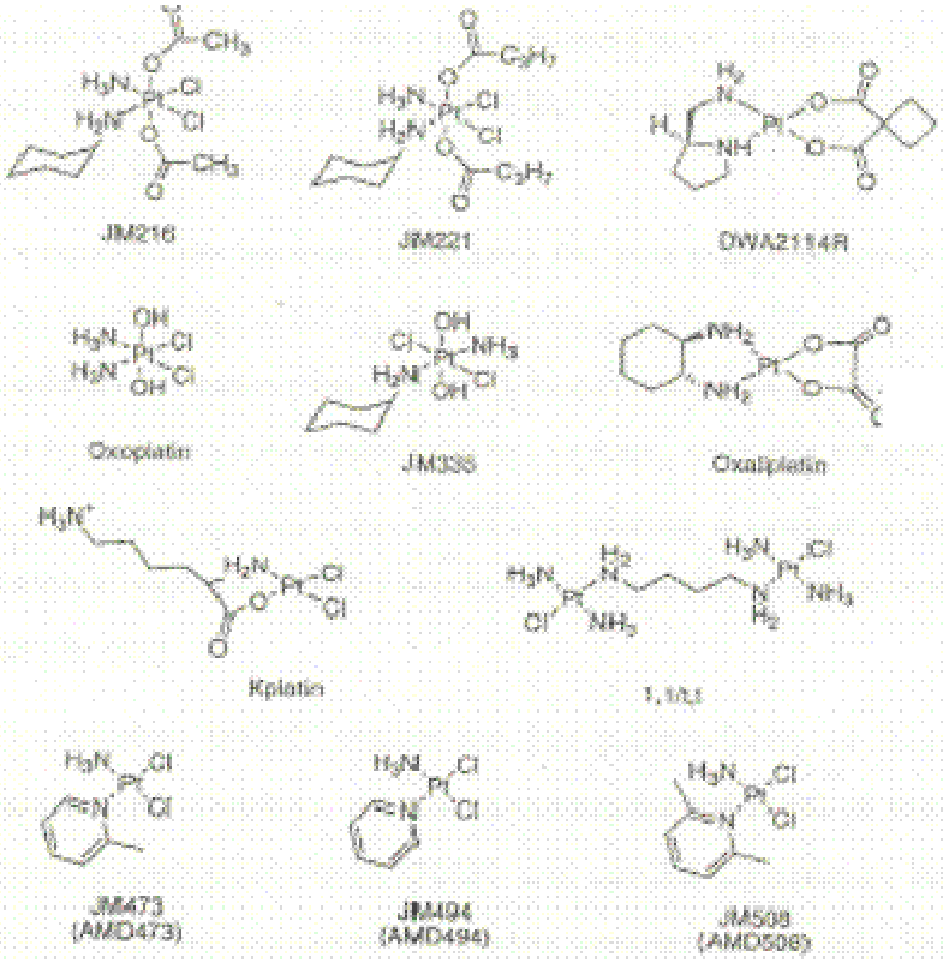


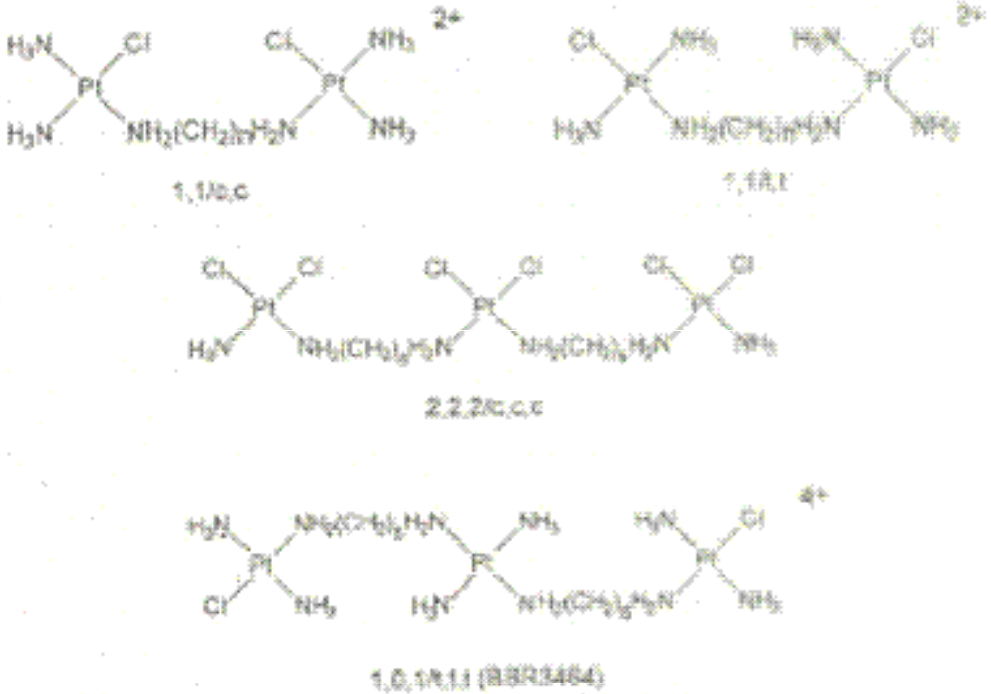
some adduct types preferred (and/or more stable than others)

1,2-GG and 1,2-AG IACs = the main cytotoxic lesions



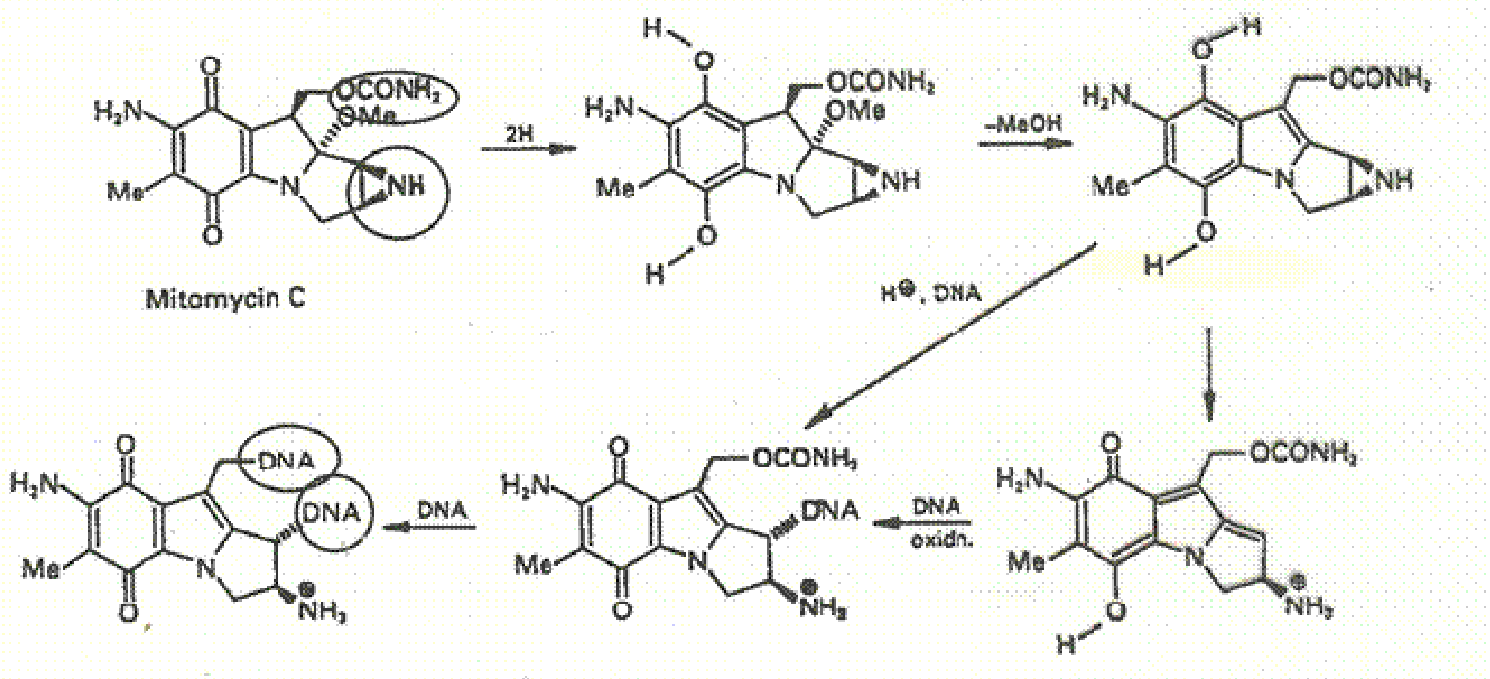
other platinum complexes tested as cytostatics





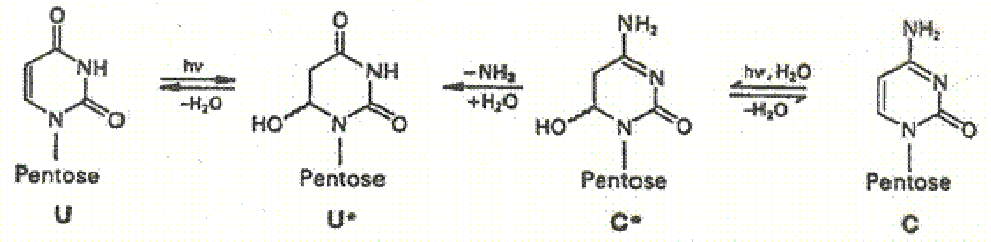
mitomycin C

- reactive aziridine group, quinone group
- reductive activation
- bifunctional adducts

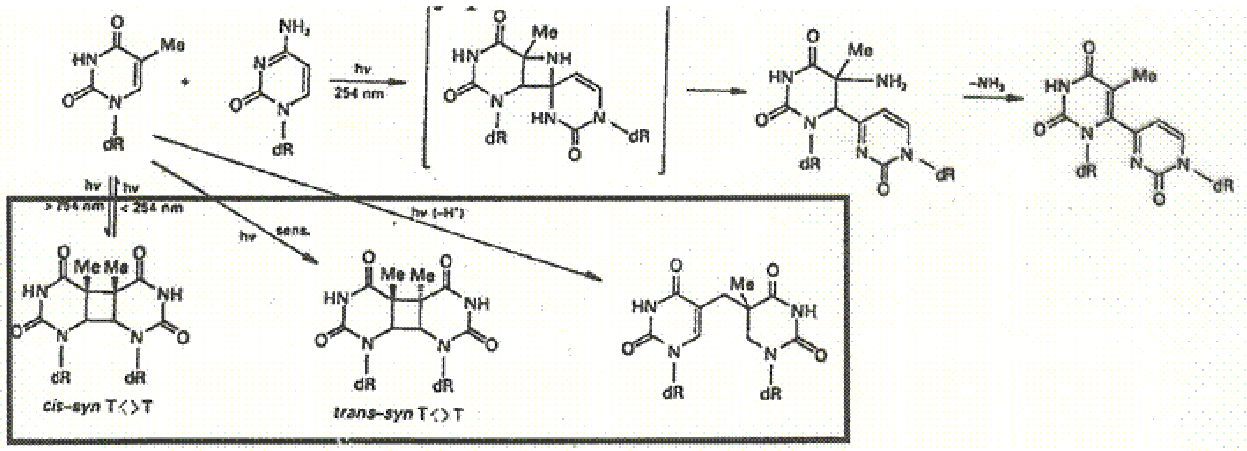


Photochemical DNA modifications

- mainly pyrimidines
- excitation at 240-280 nm: reactive singlet state
- water addition at C5-C6



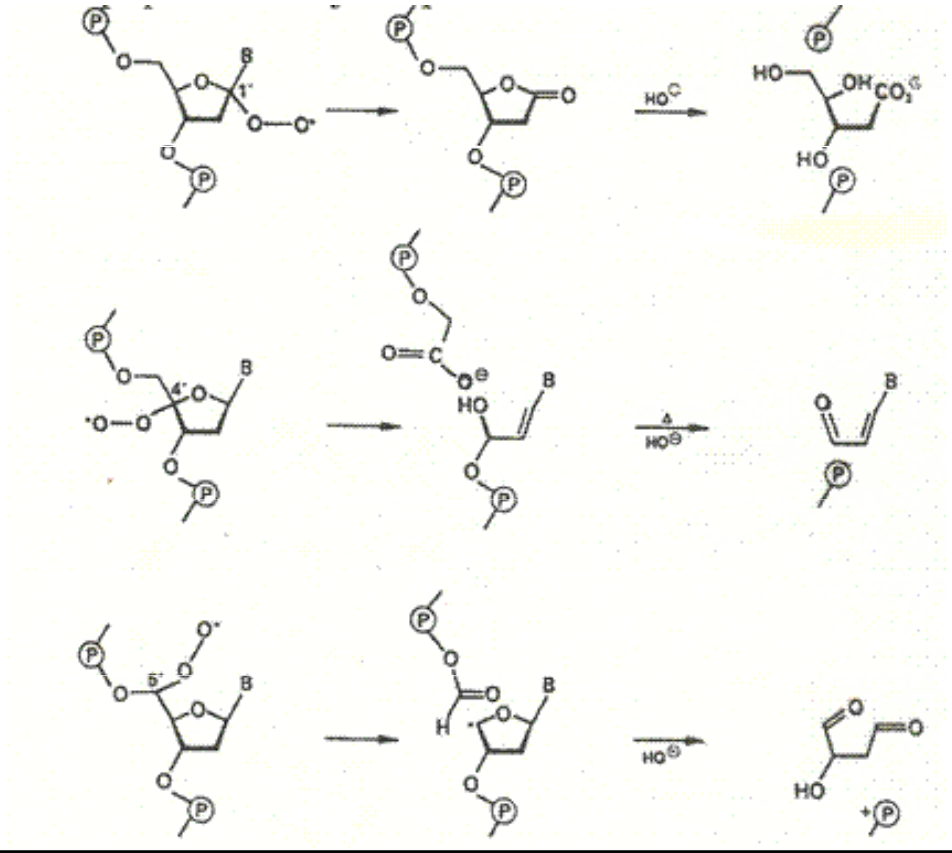
- excitation at 260-280 nm: photodimerization of pyrimidines



- photoproducts of C can deaminate to U (mutagenic effects)

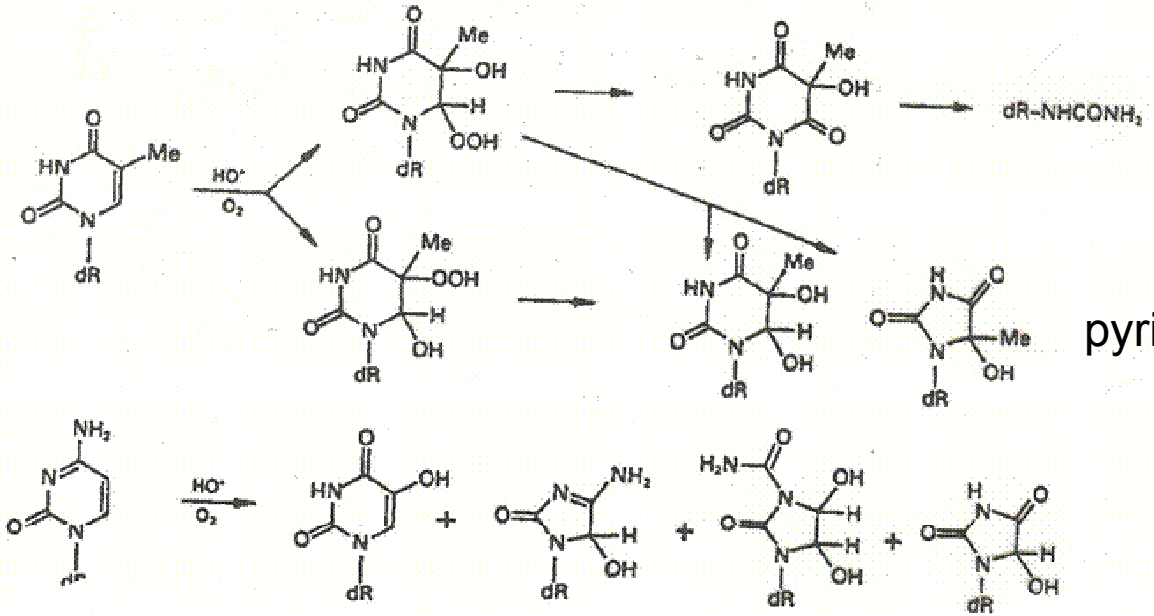
effects of ionizing radiation

- mostly indirect – through water radiolysis
- each 1,000 eV produces ~27 •OH radicals that attack DNA
- sugar damage:abstraction of hydrogen atoms from C-H bonds
- a series of steps resulting in strand breakage

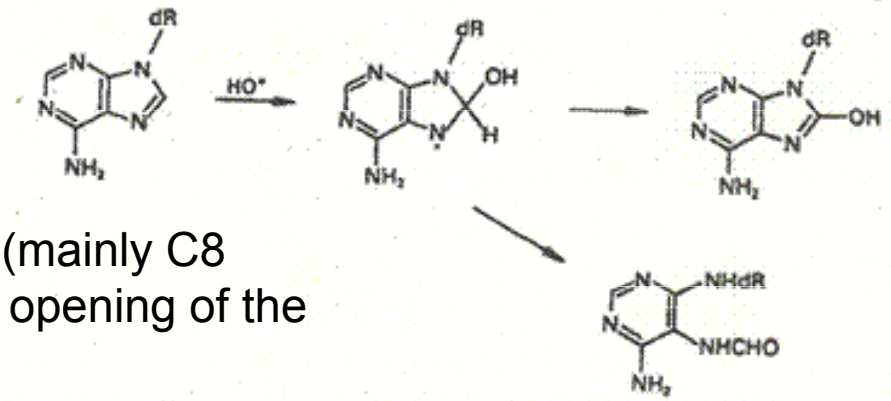


effects of ionizing radiation

- base damage: hydroxylation and/or (under aerobic conditions) peroxylation



pyrimidine products



purine products (mainly C8 hydroxylation or opening of the imidazole ring)

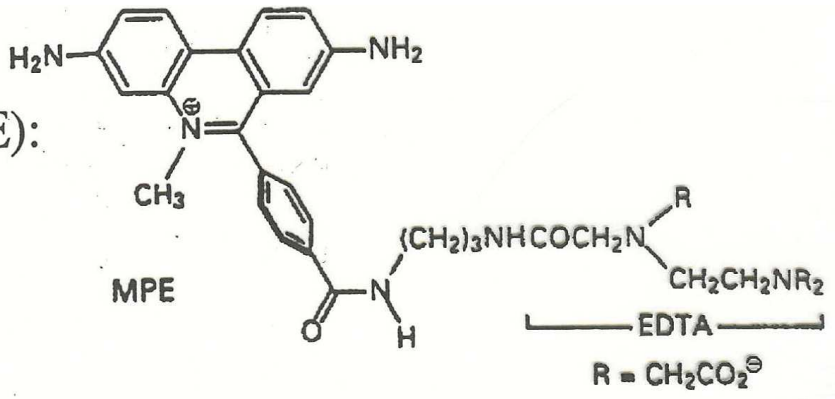
chemical nucleases

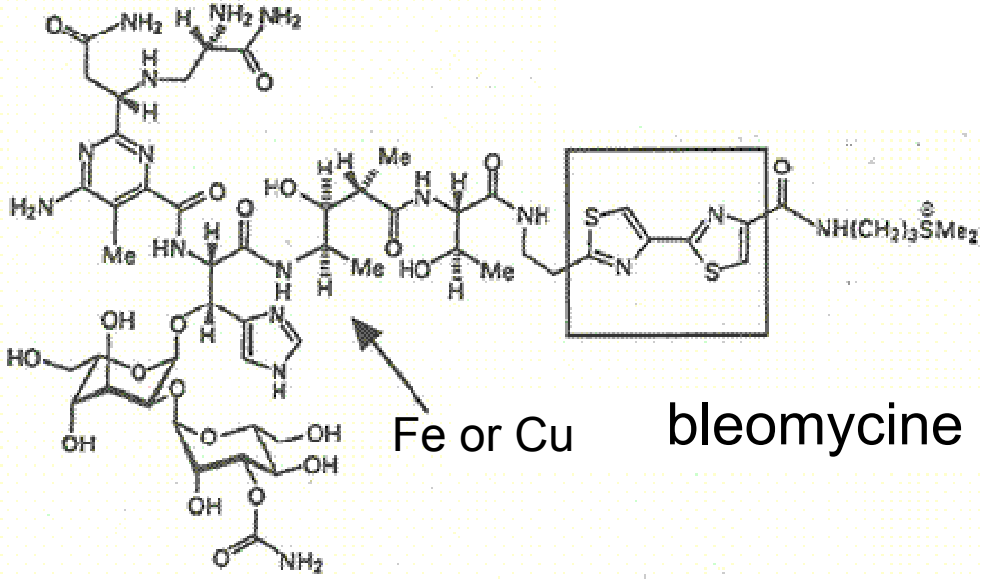
species containing redox active metal ions mediating production of hydroxyl radicals (or othe reactive oxygen species) via Fenton and/or Haber-Weiss processes

$$Me^{n} + H_2O_2 \rightarrow Me^{n+1} + \bullet OH + OH^{-}$$

iron/EDTA complex
$Cu(phen)_2$ complex

-methidiumpropyl(EDTA) (MPE):

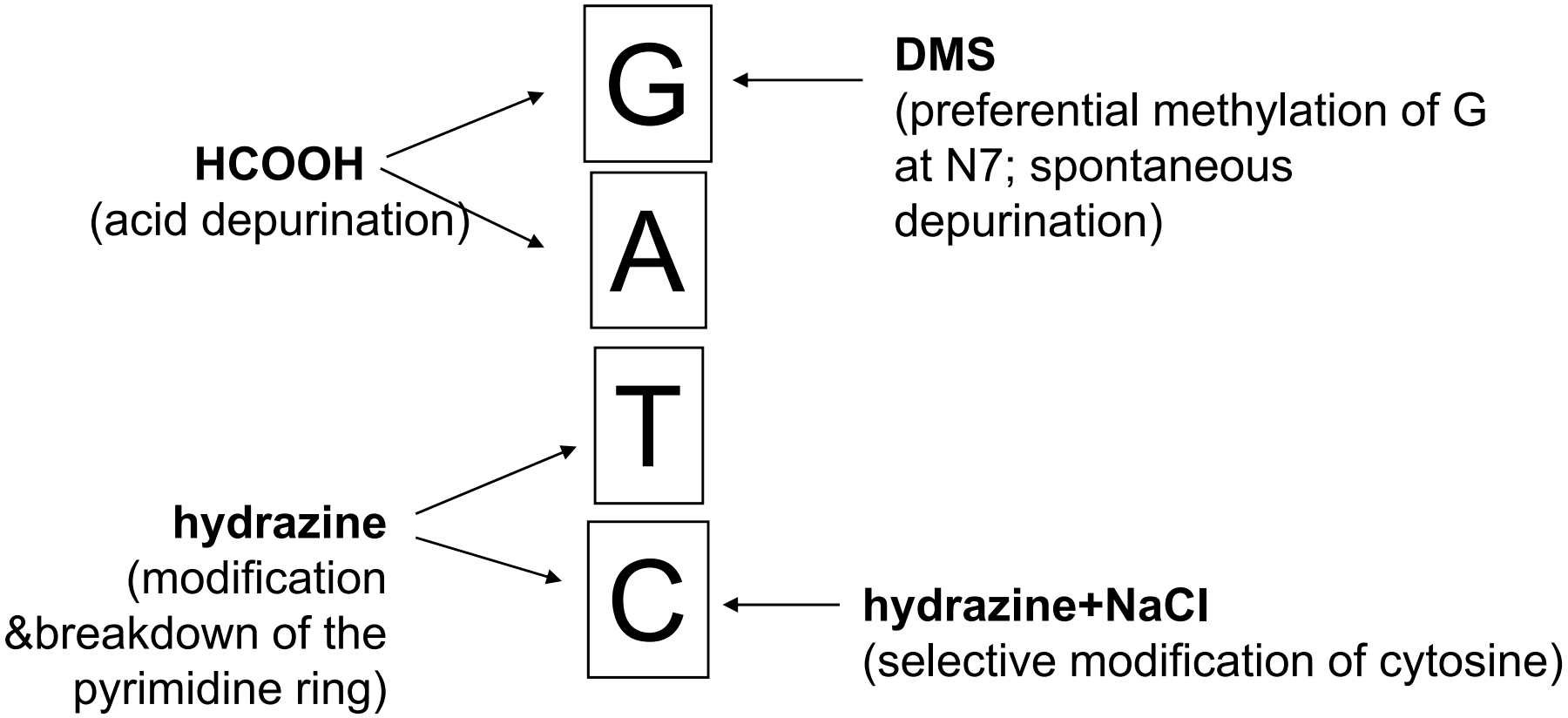




Chemical approaches in DNA studies

(several examples)

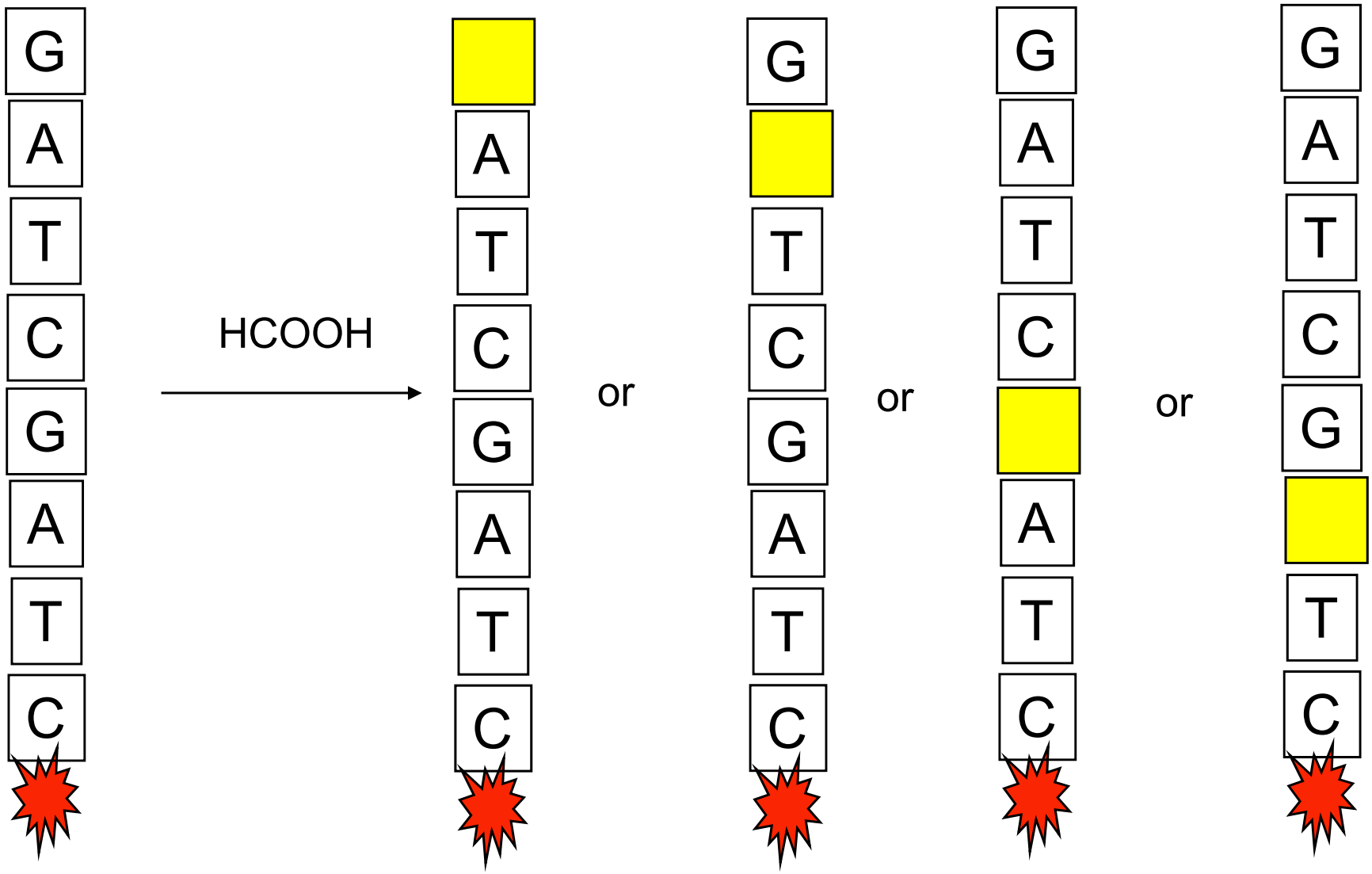
Maxam and Gilbert method of DNA sequencing

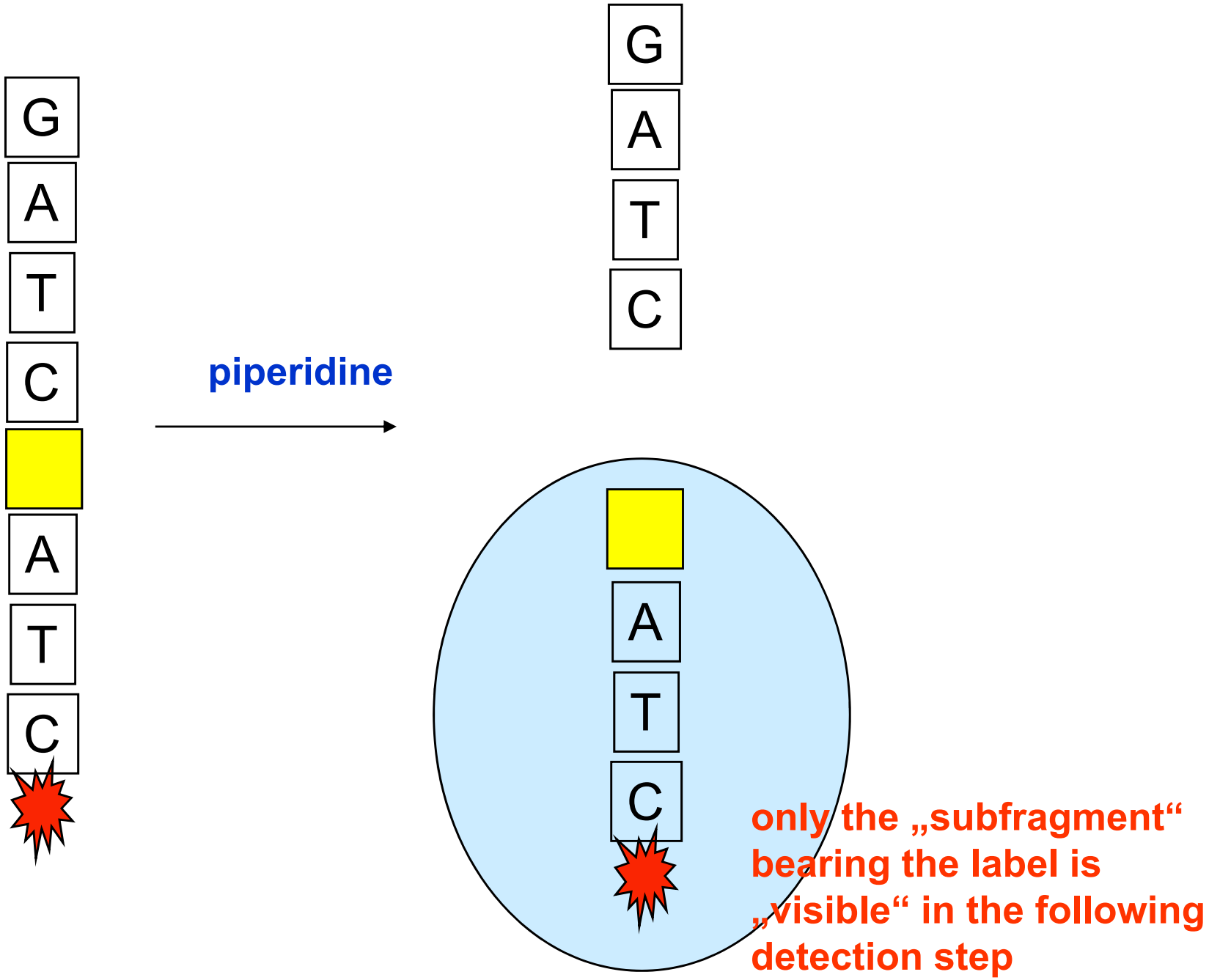


at sites of base modification (removal) the sugar-phosphate backobone is labile towards alkali

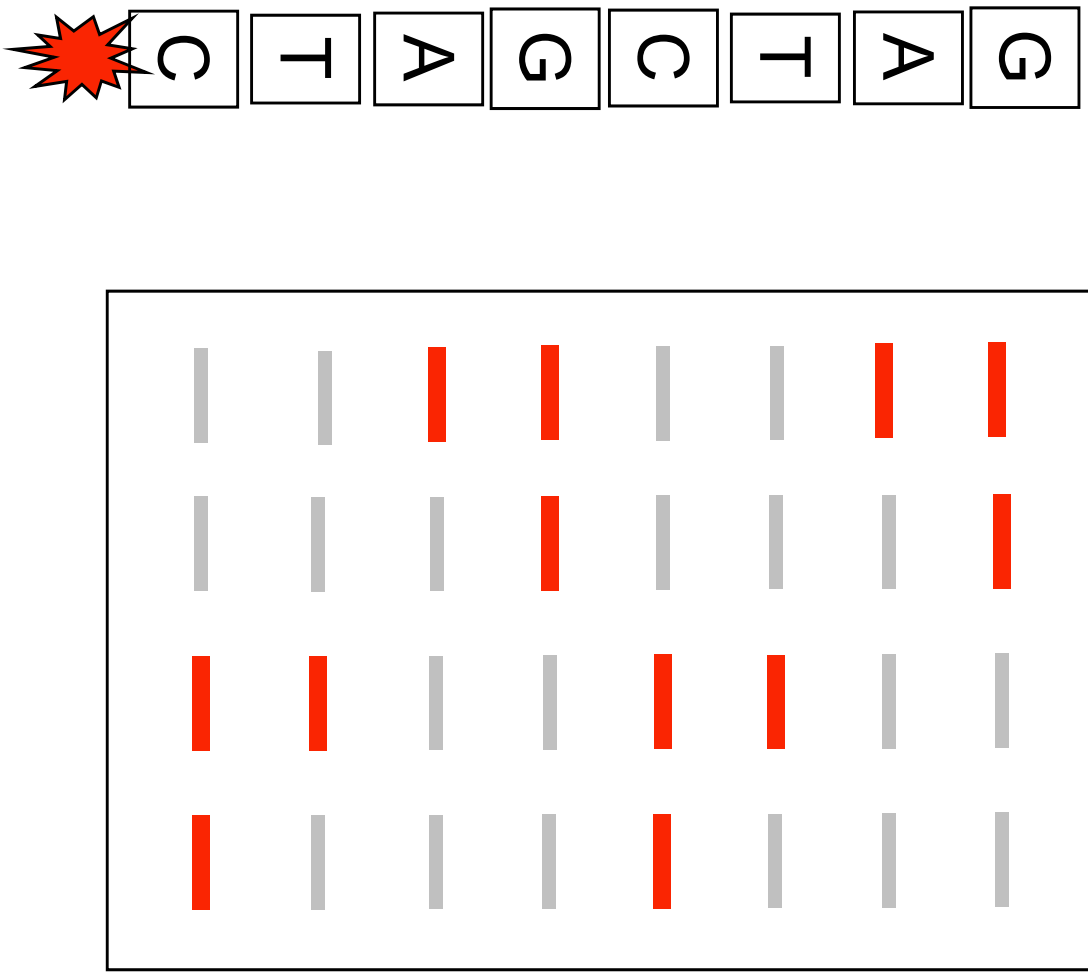
treatment with hot piperidine → cleavage at such sites

- DNA fragment is end-labeled (radionuclide, fluorophore)
- the sample is divided into four reactions (HCOOH, DMS, hydrazine, hydrazine + NaCl)
- the conditions are chosen to reach only one modification event per DNA molecule


G
A
T
C
G
A
T
C
HCOOH
A
T
C
G
A
T
C
or
G
T
C
G
A
T
C
or
G
A
T
C
A
T
C
or
G
A
T
C
G
T
C


G
A
T
C
A
T
C
piperidine
G
A
T
C
A
T
C
only the „subfragment“ bearing the label is „visible“ in the following detection step

G
A
T
C
G
A
T
C
HCOOH
piperidine
A
T
C
G
A
T
C
or
T
C
G
A
T
C
or
A
T
C
or
T
C
PAGE
autoradiography


C
T
A
G
C
T
A
G
HCOOH
DMS
hydrazine
Hydrazine
+NaCl

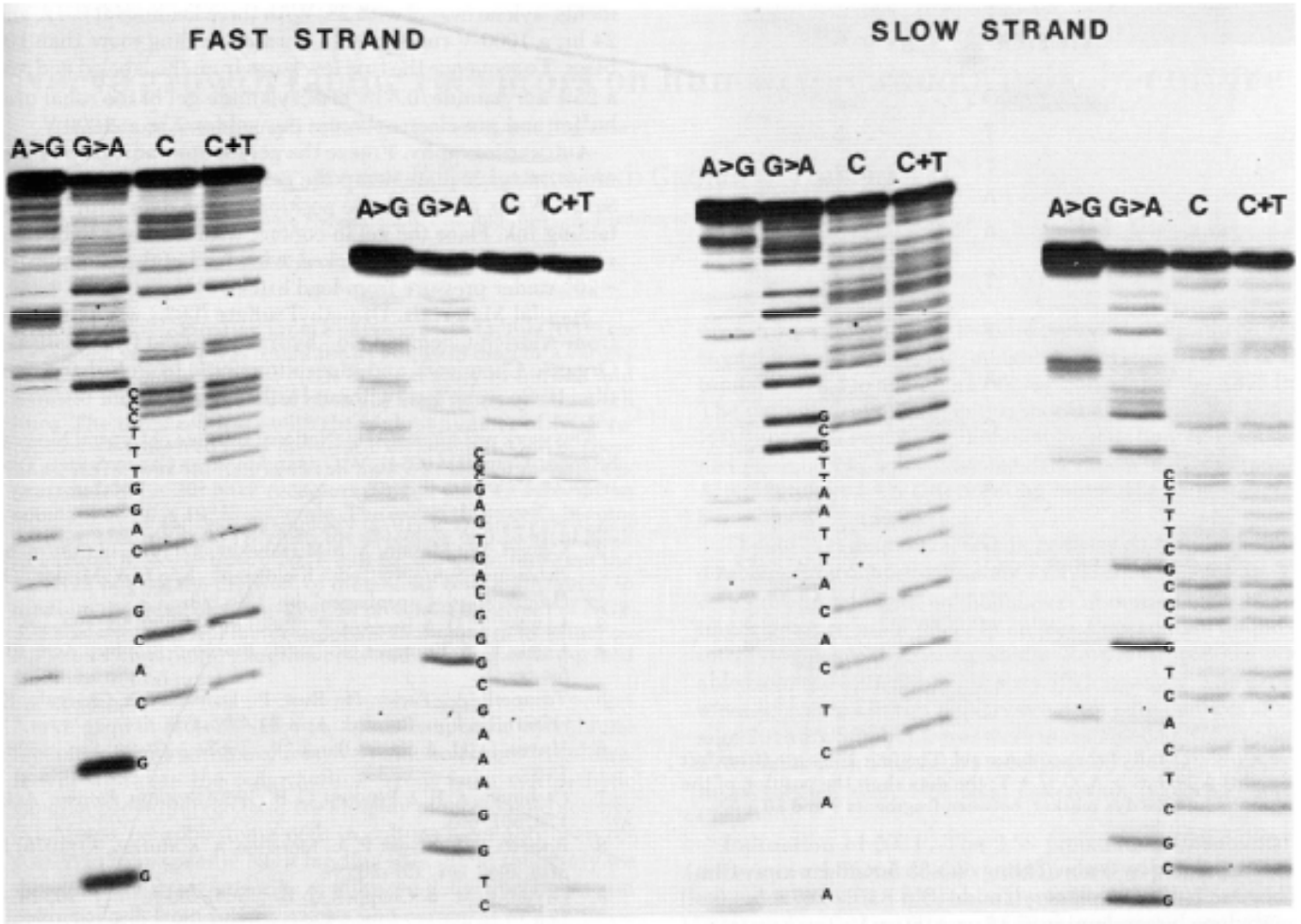
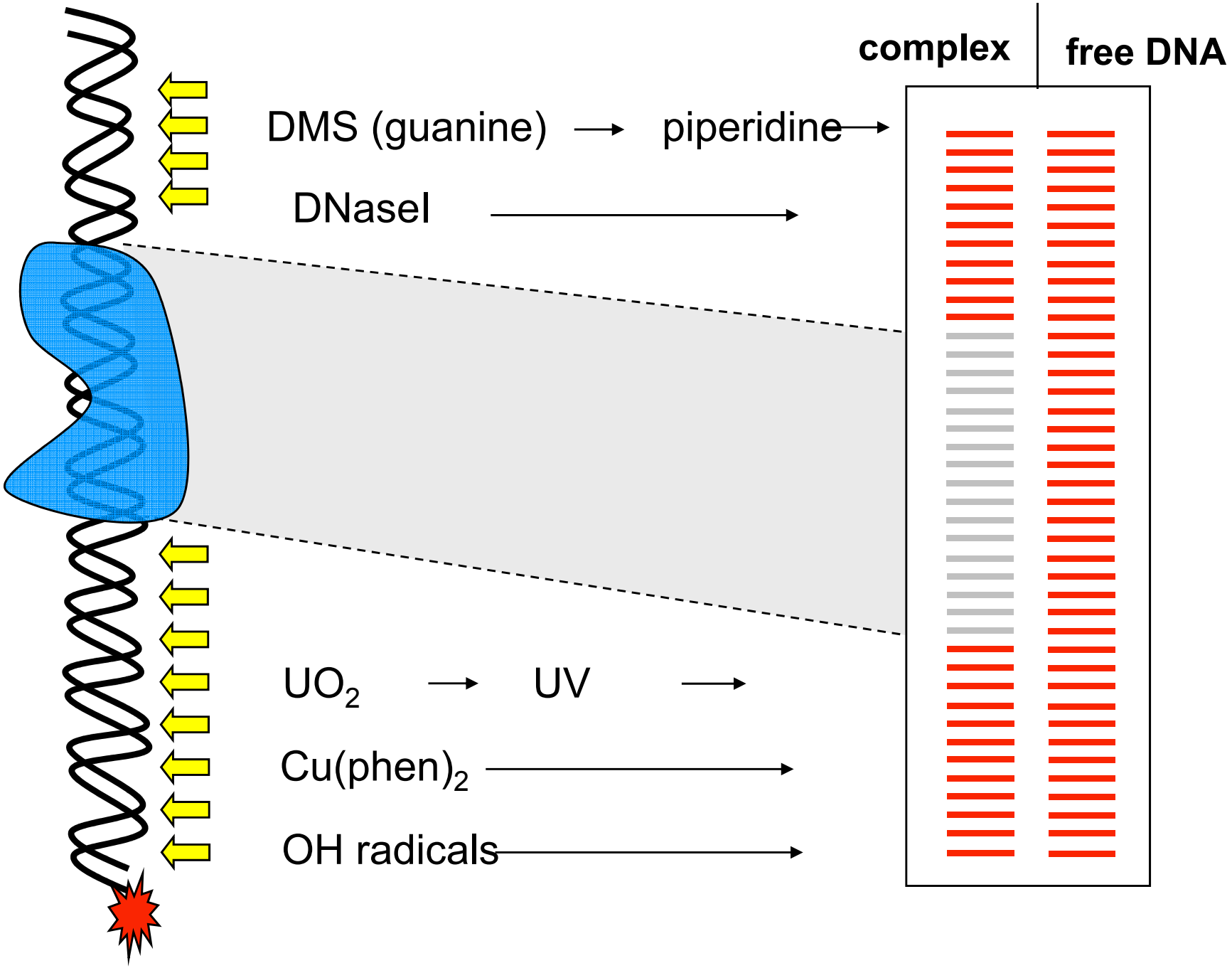


FIG. 2. Autoradiograph of a sequencing gel of the complementary strands of a 64-base-pair DNA fragment. Two panels, each with four reactions, are shown for each strand; cleavages proximal to the 5′ end are at the bottom on the left. A strong band in the first column with a weaker band in the second arises from an A; a strong band in the second column with a weaker band in the first is a G; a band appearing in both the third and fourth columns is a C; and a band only in the fourth column is a T. To derive the sequence of each strand, begin at the bottom of the left panel and read upward until the bands are not resolved; then, pick up the pattern at the bottom of the right panel and continue upward. One-tenth of each strand, isolated from the gel of Fig. 1, was used for each of the base-modification reactions. The dimethyl sulfate treatment was 50 mM for 30 min to react with A and G; hydrazine treatment was 18 M for 30 min to react with C and T and 18 M with 2 M NaCl for 40 min to cleave C. After strand breakage, half of the products from the four reactions were layered on a 1.5 × 330 × 400 mm denaturing 20% polyacrylamide slab gel, pre-electrophoresed at 1000 V for 2 hr. Electrophoresis at 20 W (constant power), 800 V (average), and 25 mA (average) proceeded until the xylene cyanol dye had migrated halfway down the gel. Then the rest of the samples were layered and electrophoresis was continued until the new bromphenol blue dye moved halfway down. Autoradiography of the gel for 8 hr produced the pattern shown.

DNA „footprinting“: determination of binding sites of other molecules (e.g. proteins) at the DNA sequence level


complex
free DNA
DMS (guanine)
piperidine
DNaseI
UO_2
UV
$Cu(phen)_2$
OH radicals

single strand-selective chemical probes

Open local structures in negatively supercoiled DNA

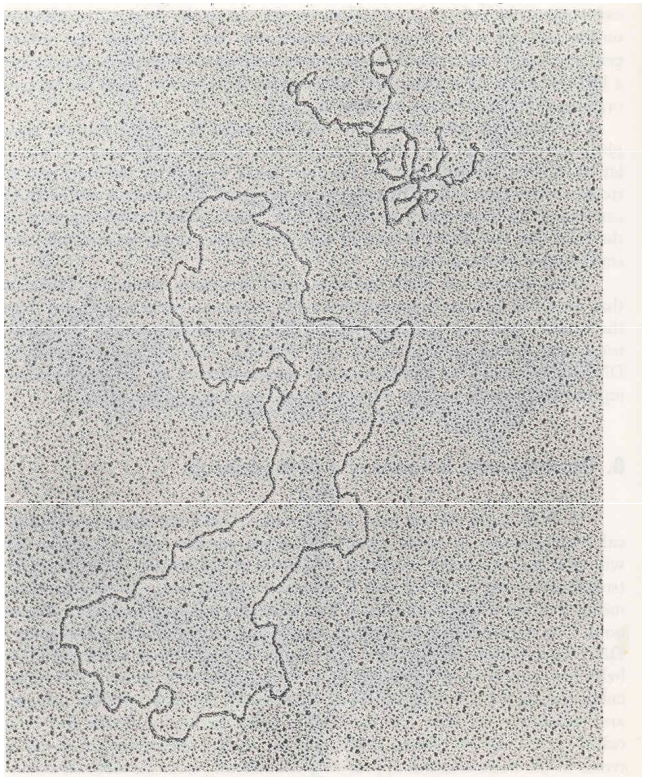
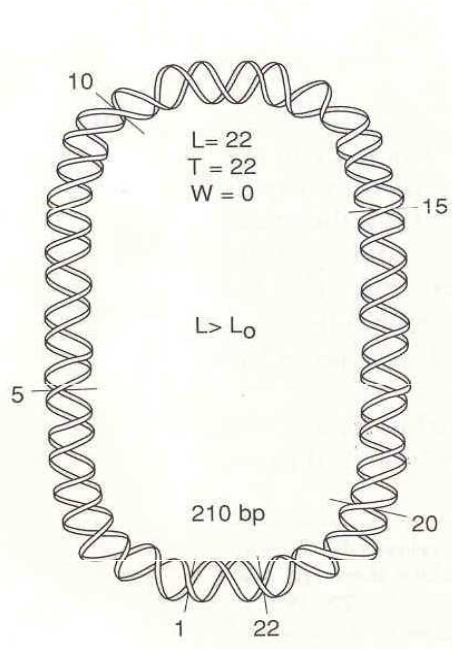
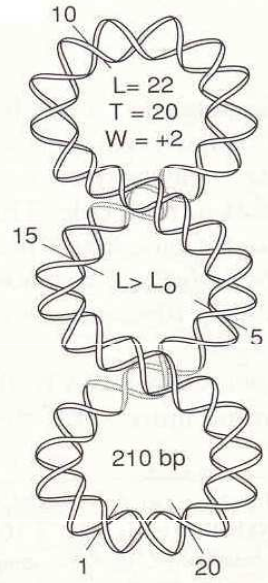


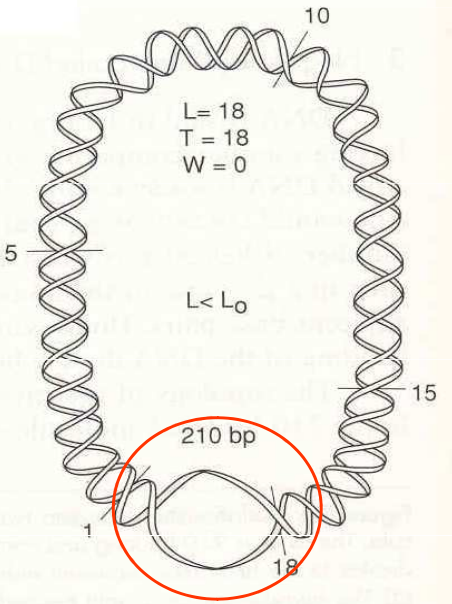
Figure 3.2 Electron micrograph of two forms of DNA. The tangled, twisted molecule is supercoiled DNA, originally called Form I DNA. When circular molecules are relaxed (or nicked) (Form II DNA), they lose the twists. A linear molecule (not shown) is called Form III. The plasmid molecules shown are 9000 bp in length. Courtesy of Jack D. Griffith.



relaxed circular DNA



negatively supercoiled DNA (linking deficit)

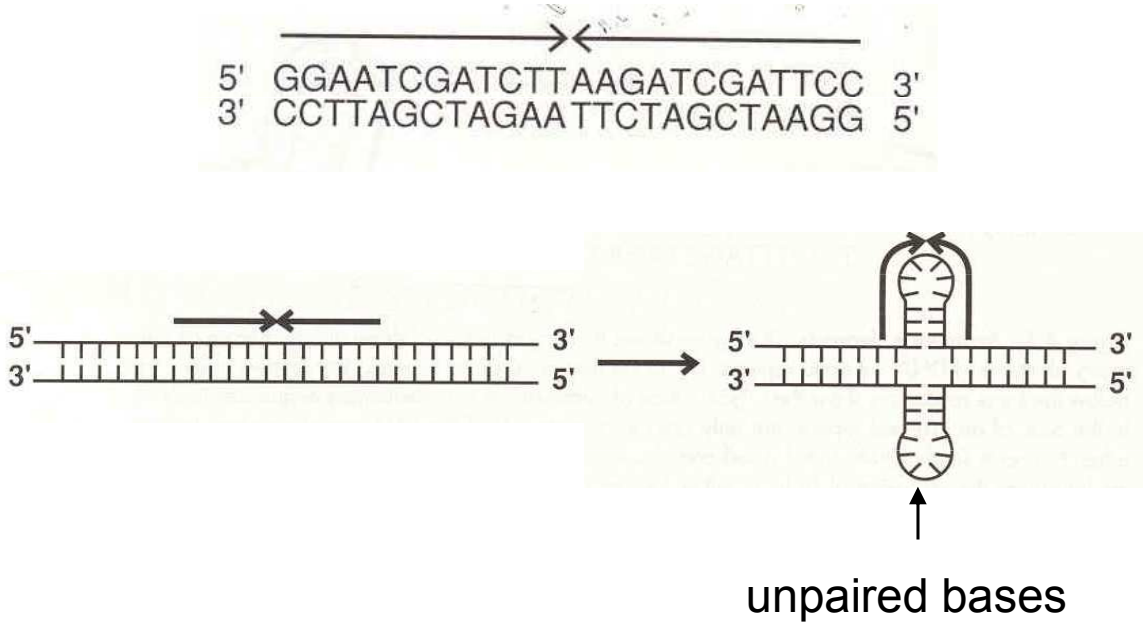


stress related to the negative superhelicity (the linking deficit) can be absorbed in local open structures

Open local structures in negatively supercoiled DNA

DNA segments of specific sequence can adopt „alternative“ local structures

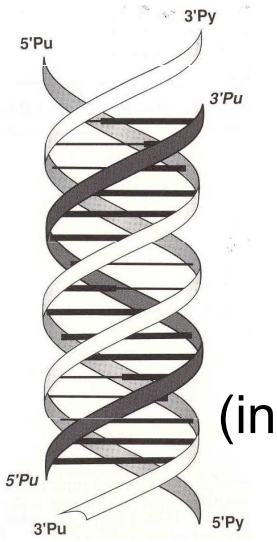
cruciform DNA (inverted repeat)



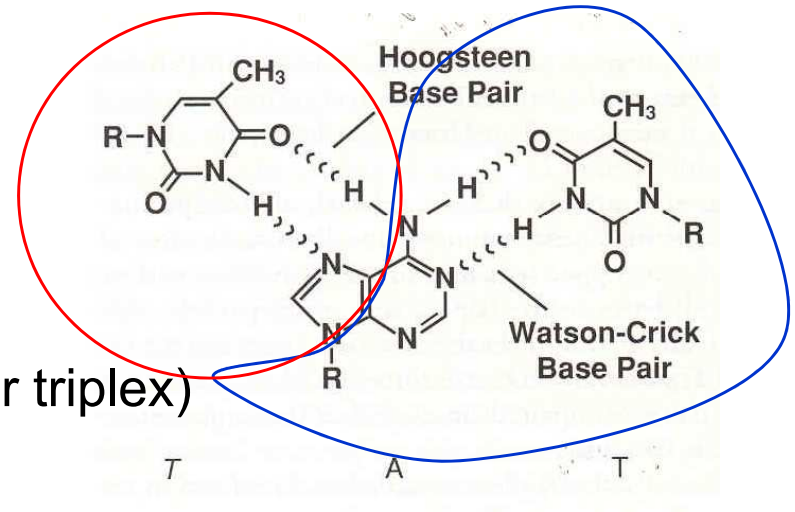
Open local structures in negatively supercoiled DNA

Triplex DNA
(homopurine·homopyrimidine stretch with mirror symmetry)

e.g. TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT



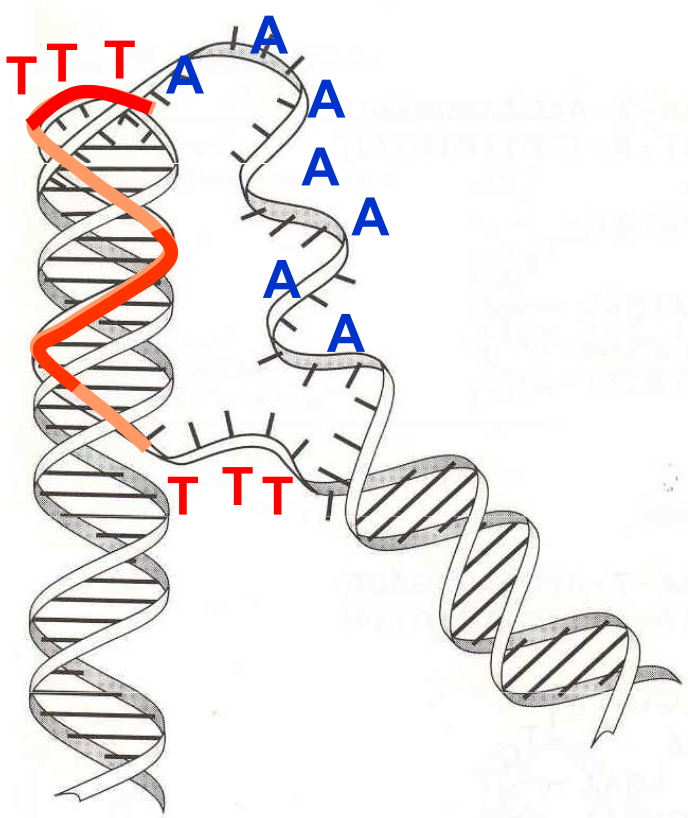
(intermolecular triplex)

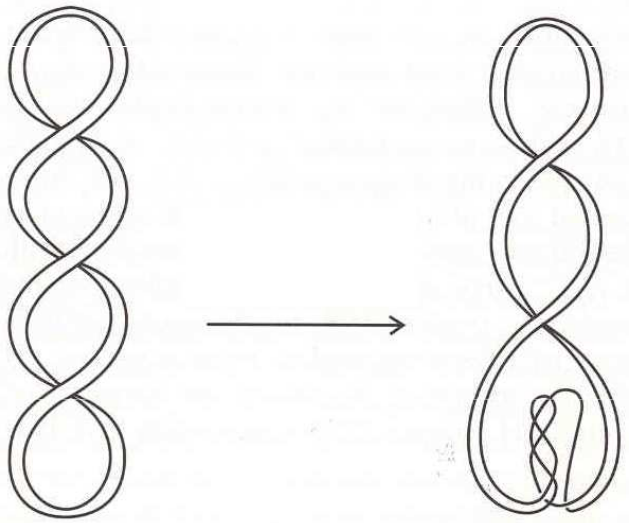


Otevřené lokální struktury v negativně nadšroubovicové (sc) DNA

Intramolecular triplex

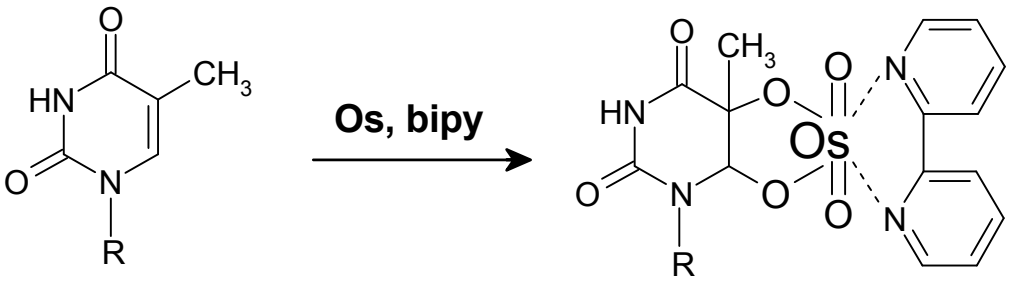
(homoPu•homoPy segment within negatively supercoiled DNA)



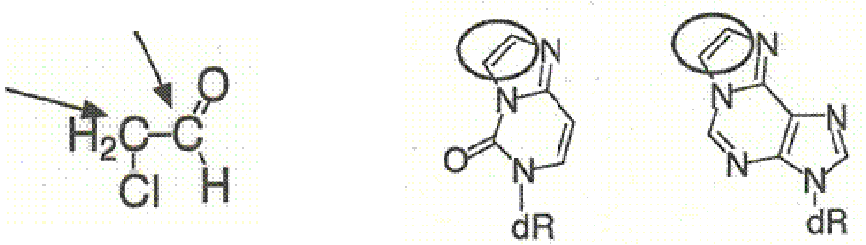


Chemicals selectively reacting with unpaired bases:

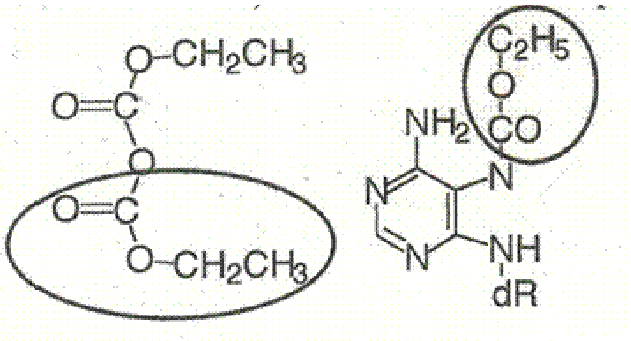
osmium tetroxide complexes
(Os,L)
(T, more slowly C)

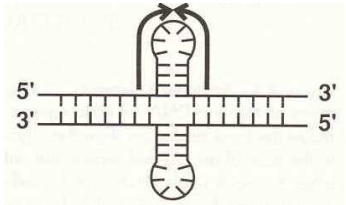


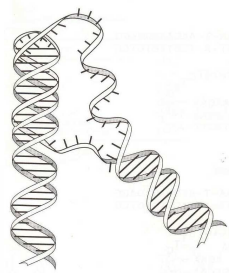
chloroacetaldehyde
(CAA)
(A, C)

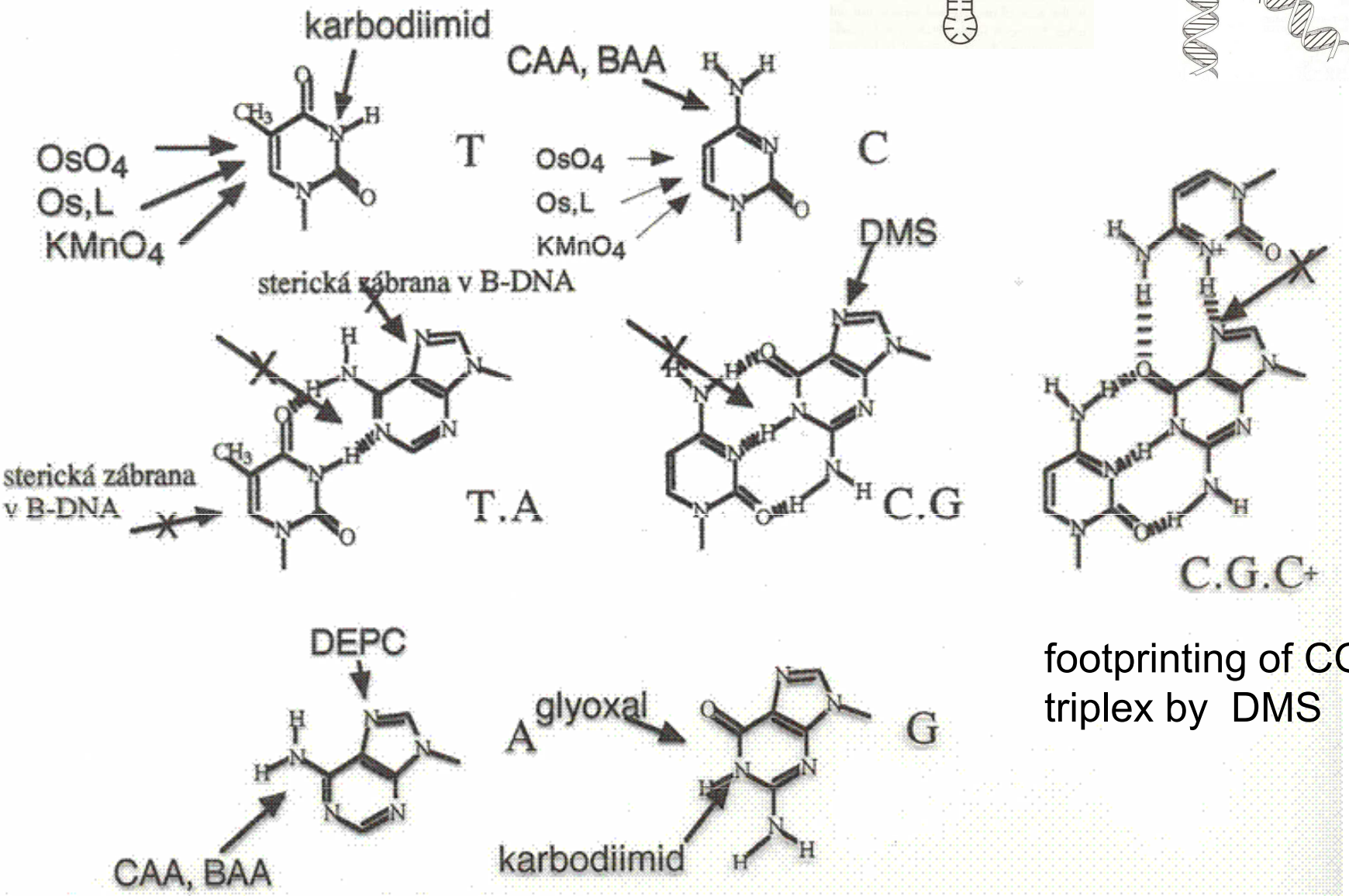


diethyl pyrocarbonate
(DEPC)
(A, G)







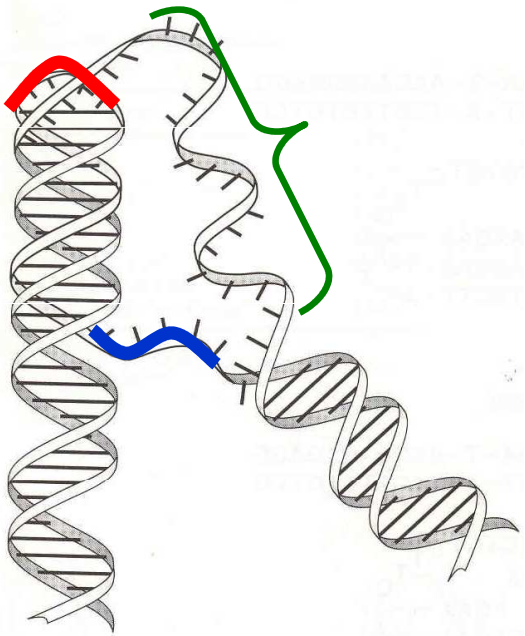


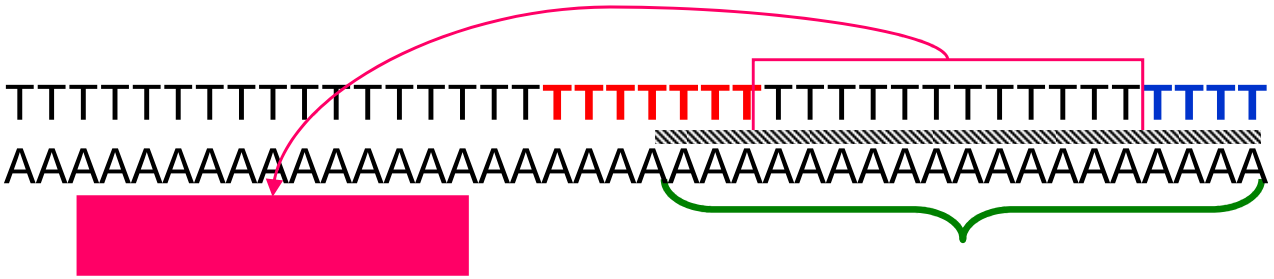
footprinting of CGC+ triplex by DMS

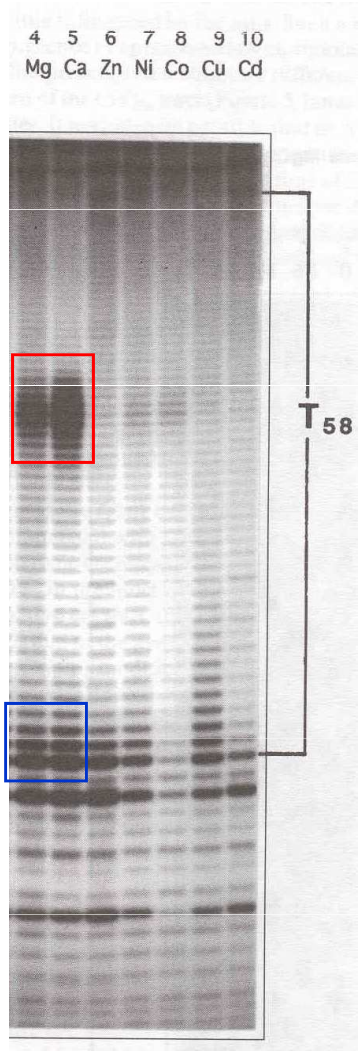
Using the Maxam-Gilbert technique, it is possible to determine with a high preciseness which nucleotides are forming the local structure

- modification of supercoiled DNA
- restriction cleavage, radiactive labeling
- hot piperidine
- sequencing PAGE

the structure can be deduced from the modification pattern







DNA damage and repair

Why is it important to study „DNA damage“?

DNA: the genetic material ensuring

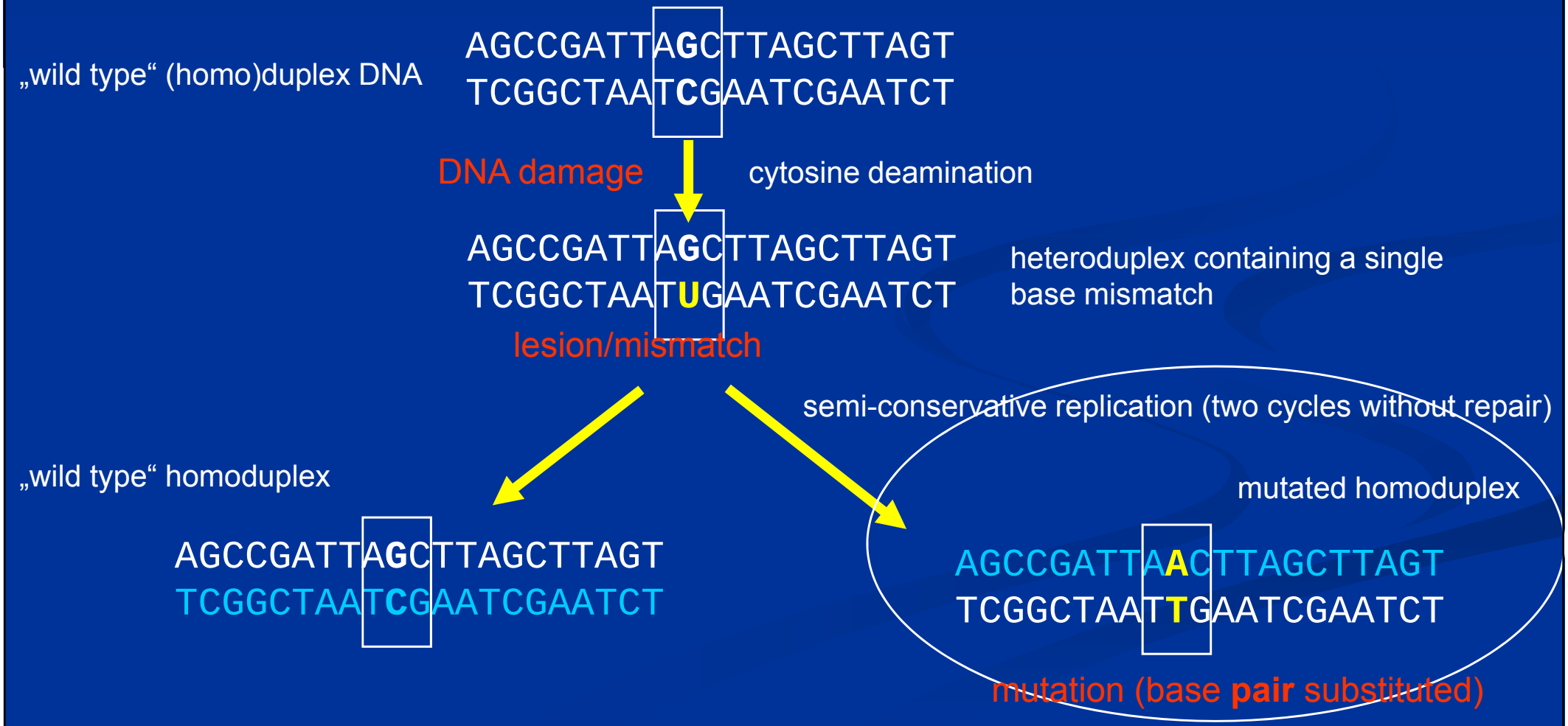
- preservation of the genetic information
- its transfer to progeny
- its transcription and translation into proteins

Damage to DNA may

- lead to change of the genetic information (mutation)
- affect gene expression
- **have severe health impacts**

DNA damage, mutation, lesion, mismatch…?

- mutation may arise from (among others) DNA damage which is not repaired prior to DNA replication, e.g..

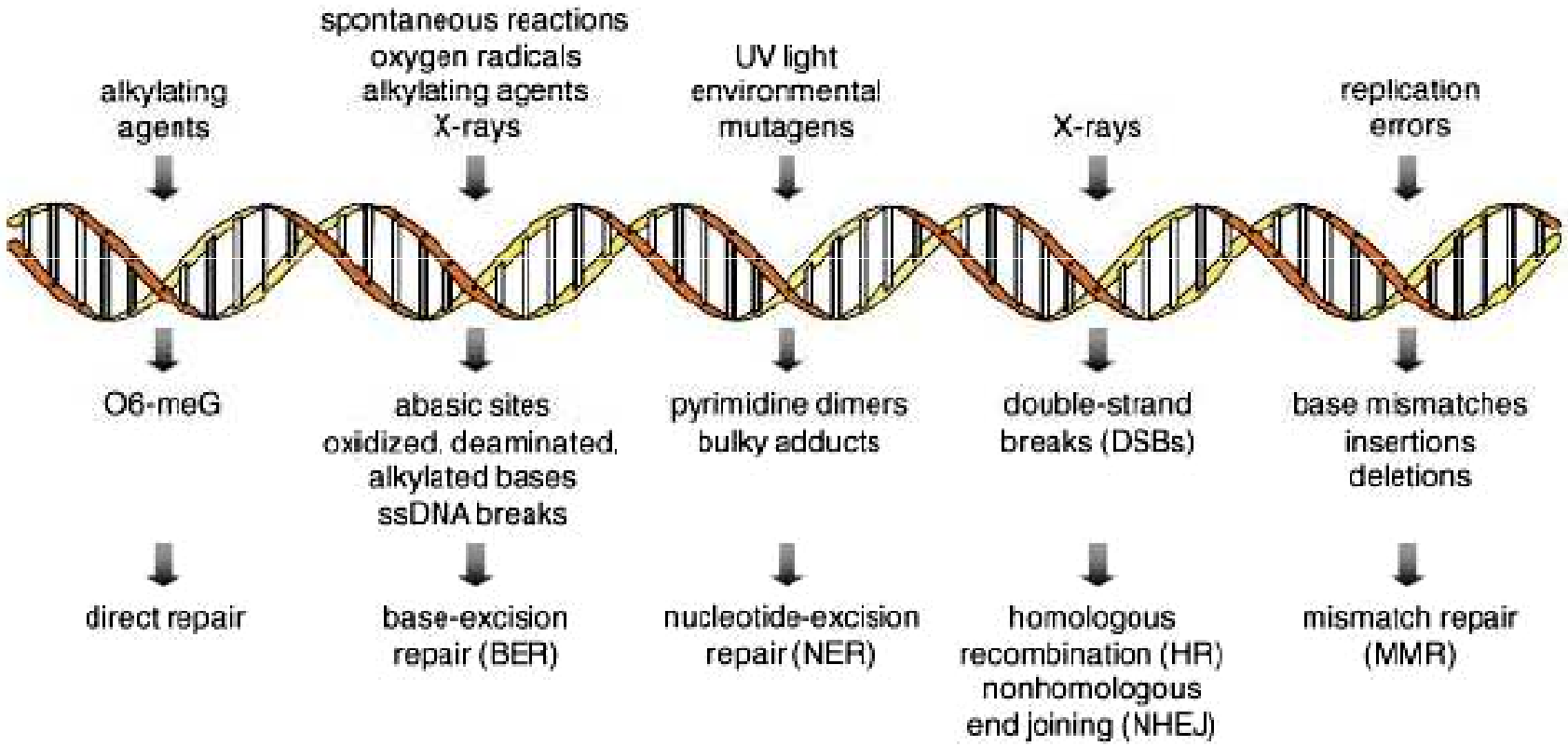


DNA damage, mutation, lesion, mismatch…?

- **mutations arise from unrepaired DNA damage** (or from replication errors)
- **damaged DNA is not mutated yet!** (damage is usually repaired in time i.e. before replication – lesions and/or mismatches are recognized by the reparation systems)
- **DNA with mutated nucleotide sequence does not behave as damaged!** All base pairs in such DNA are „OK“ (no business for the DNA repair machinery) but the **genetic information is** (hereditably) **altered.**

DNA in the cells is permanently exposed to various chemical or physical agents

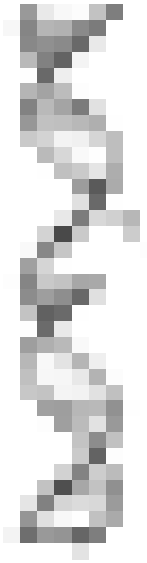
- endogenous - products and intermediates of metabolism
- exogenous - environmental (radiation, pollutants)

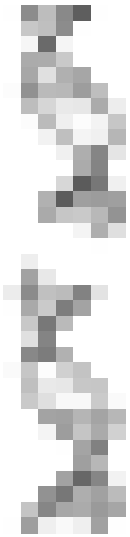


Scharer, O. D. (2003) Chemistry and biology of DNA repair, *Angew. Chem. Int. Ed. 42*, 2946-74.

Most frequent products of DNA damage („lesions“)

interruptions of DNA sugar-phosphate backbone



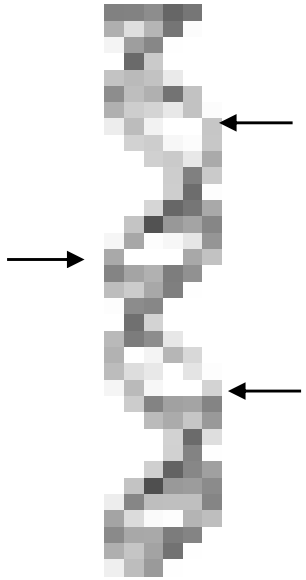


single-strand break

double-strand break

- reactive oxygen species
- action of nucleases
- consequence of base damage

interruption of the N-glykosidic linkage



abasic sites

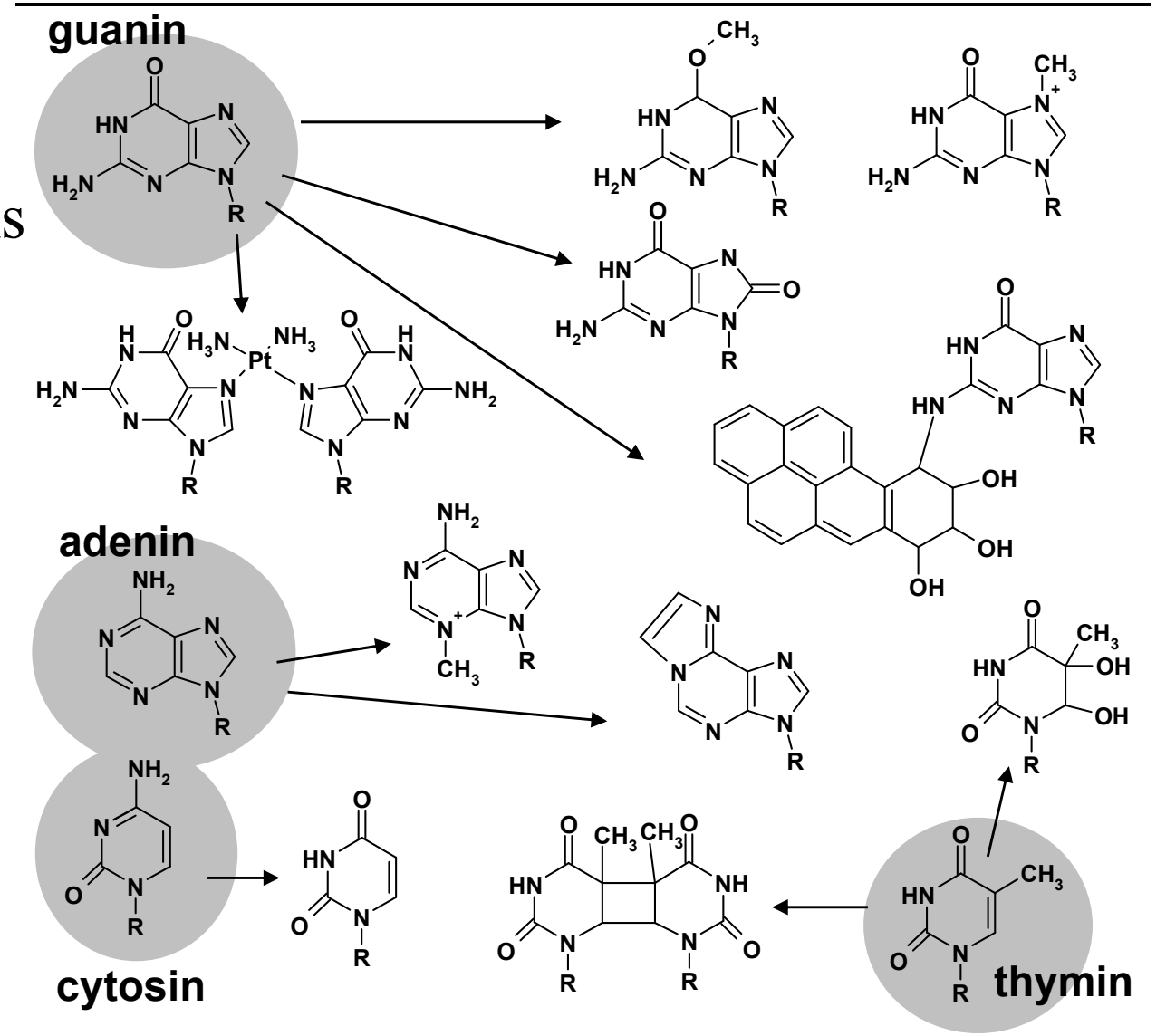
- spontaneous hydrolysis (depurination)
- consequence of base damage

Most frequent products of DNA damage („lesions“)

base damage:

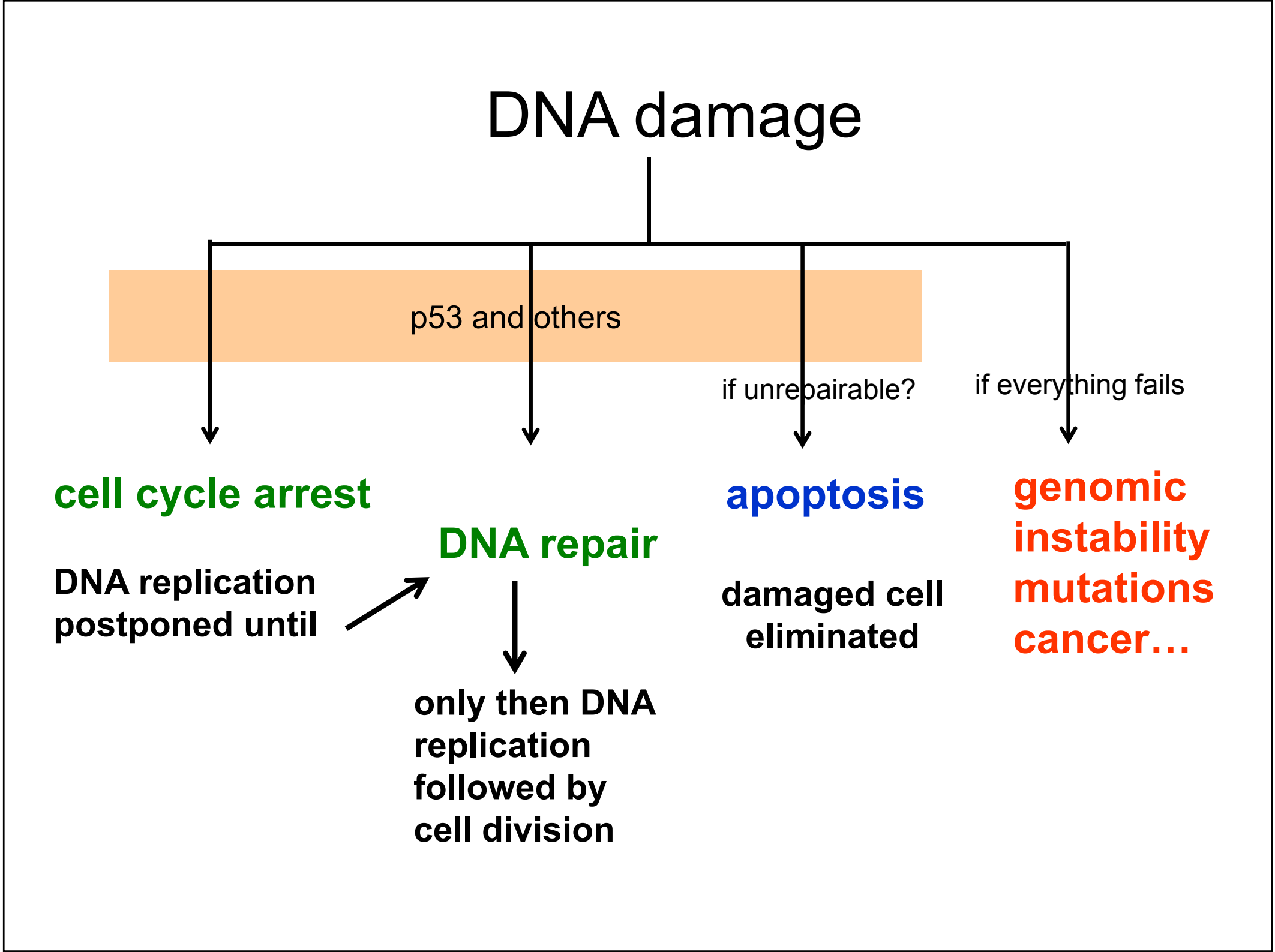
chemical modifications

- **alkylation**
- **oxidative damage**
- **deamination**
- **damage by UV radiation (sunlight)**
- **metabilically activated carcinogens**
- **anticancer drugs**



Importance of DNA repair

- estimated number of DNA-damage events in **a single human cell: 10^4-10^6 per day!!**
- only a small number of base pairs alterations in the genome are in principle sufficient for the induction of cancer
- DNA-repair systems must effectively counteract this threat
- in an adult human (10^{12} cells) about 10^{16}–10^{18} repair events per day

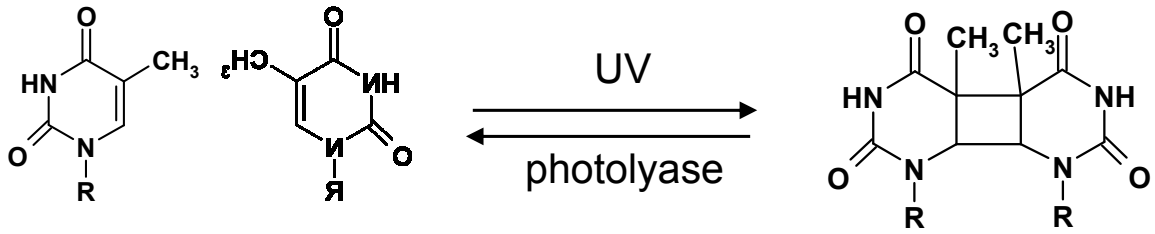

DNA damage
p53 and others
if unrepairable?
if everything fails
cell cycle arrest
DNA repair
apoptosis
genomic instability mutations cancer…
DNA replication postponed until
damaged cell eliminated
only then DNA replication followed by cell division

DNA repair pathways

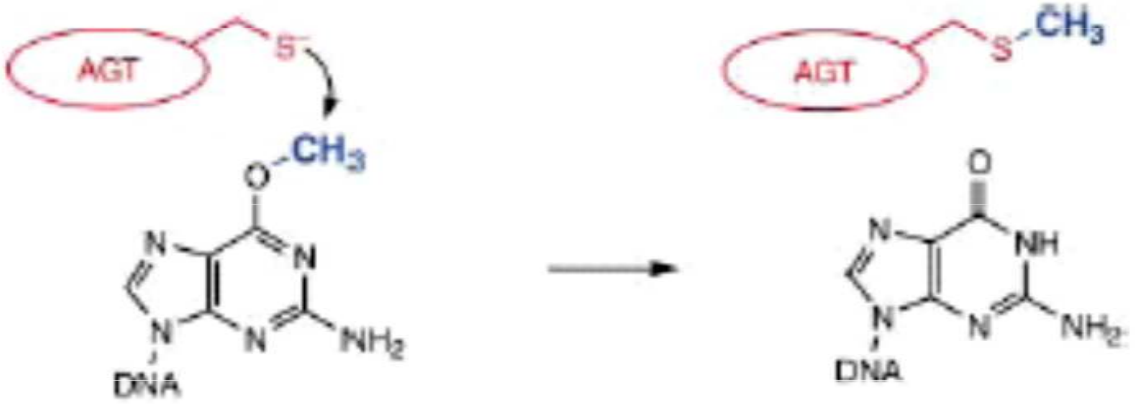
- direct reversal of damage
- base excision repair
- nucleotide excision repair
- mismatch repair
- repair of double strand breaks

Direct reversal of DNA damage

- photolyases: repair of cyclobutane dimers



- O6-alkylguanine transferase: reverses O6-alkylguanine to guanine by transferring the alkyl group from DNA to a reactive cysteine group of the protein

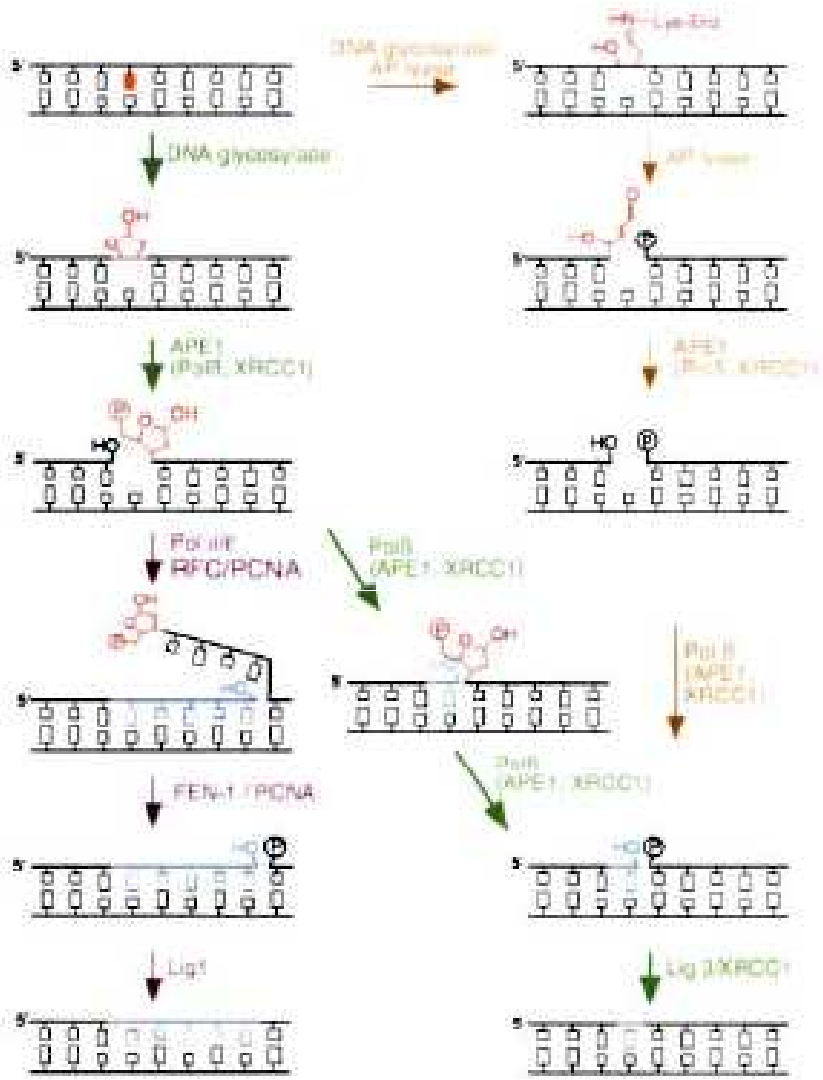


Base excision repair

- repair of damage by deamination (U, I), oxidation (8-oxoG), and alkylation
- initiated by **DNA glycosylases**, which recognize damaged bases and excise them from DNA by hydrolyzing the N-glycosidic bond
- substrate specificity of the glycosylases: developed to repair expectable „errors“?

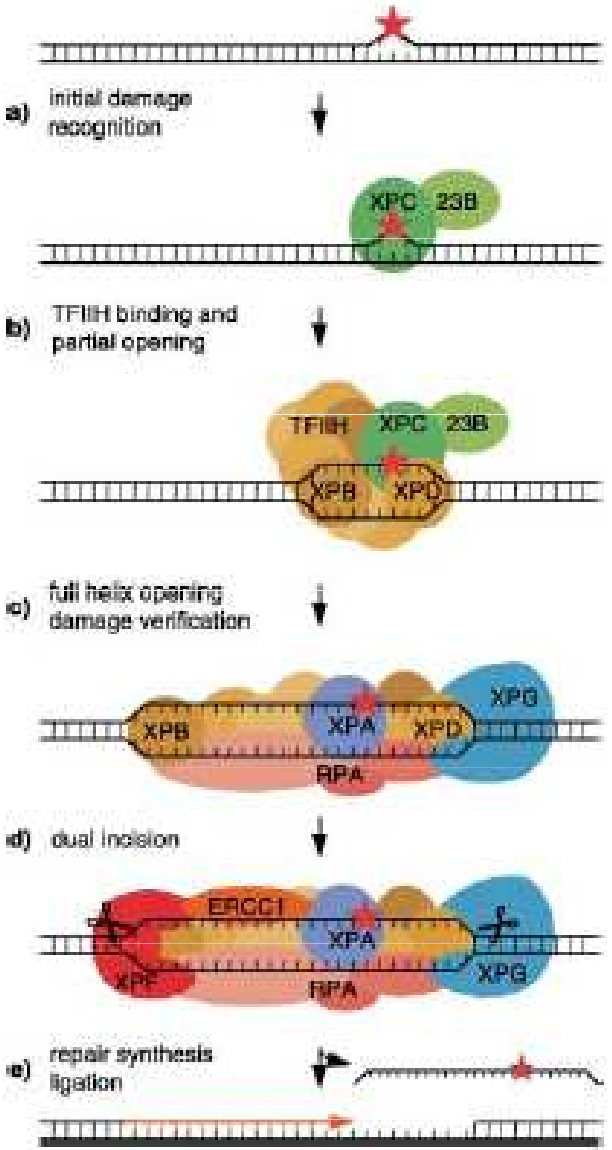
Table 1: Human DNA glycosylases

Enzyme	Most important substrate	AP lyase
UNG	U, 5-OH-U in ss/dsDNA	no
SMUG1	U, 5-OH-U in ss/dsDNA	no
TDG	U:G, T:G, εC	no
MBD4	U:G, T:G	no
OGG1	8-oxoG:C, fapy	yes
MYH	A:8-oxoG	no
NTH1	ox. pyrimidine, fapy	yes
NEI1	ox. pyrimidine, fapy	yes
AAG (MPG)	3-MeA, 7-MeG, εA, Hx	no



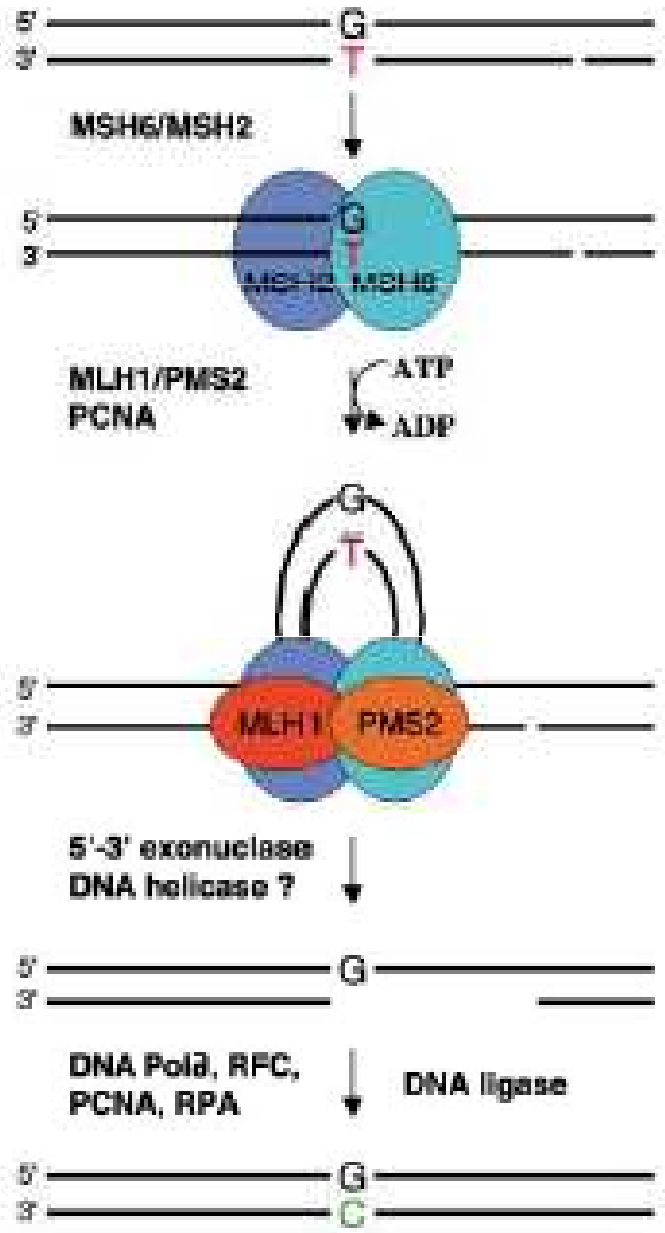
Nucleotide excision repair

- removes **bulky base adducts** (such as those formed by UV light, various environmental mutagens, and certain chemotherapeutic agents) from DNA
- broad substrate specificity: dealing with unexpected environmental DNA damaging agents
- excision of the damaged oligonucleotide
- then filling the gap & the sealing break



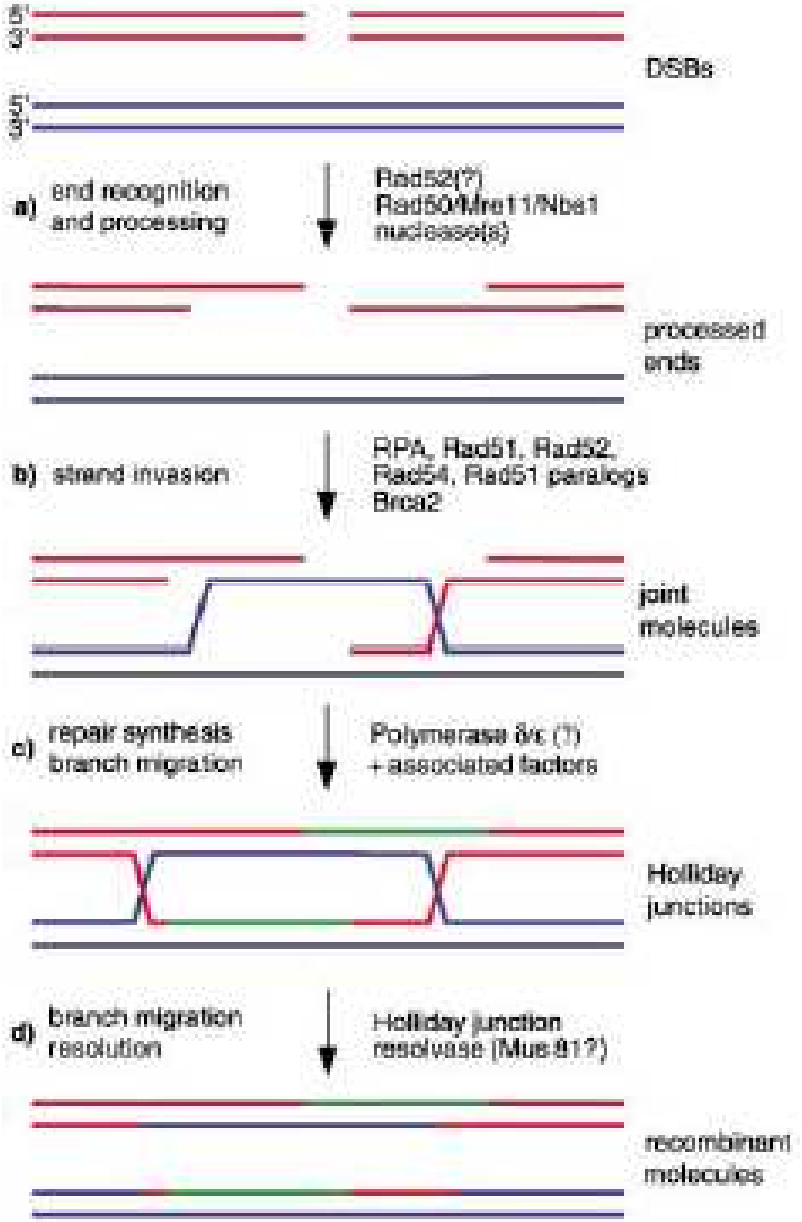
Mismatch repair

- dealing with **replication errors**
- polymerases introduce about one erroneous nucleotide per 10^5 nucleotide; their 3'→5'-exonuclease activity decreases incidence of the errors to $1:10^7$
- the MMR contributes to replication fidelity by a factor of 10^3 by removal of base-base mismatches, insertions and deletions (hence the resulting incidence of mutations due to erroneous replication is only $1:10^{10}$)
- the system must be able discrimitate between parental and daughter DNA strand!
- MutS binds to mismatches and insertion/deletion loops
- „repairosome" formation, removal of a part of the daughter strand by 5'→3'- exonuclease
- new DNA synthesis and ligation



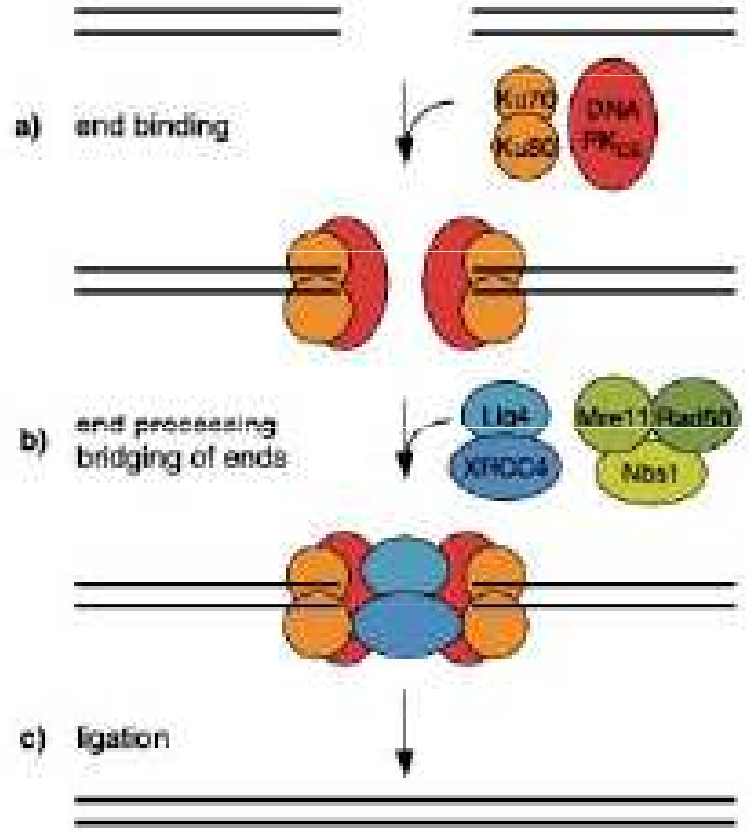
Repair of double strand breaks

- consequences of DSBs can be very severe (chromosome aberrations)
- two repair pathways:
- homologous recombination: an intrinsically accurate repair pathway that uses regions of DNA homology (such as sister chromatids) as coding information.



Repair of double strand breaks

- consequences of DSBs can be very severe (chromosome aberrations)
- two repair pathways:
- non-homologous end joining: conceptually simple pathway that involves the religation of broken ends (without using a homologous template
- less accurate: may loss of a few nucleotides at the damaged DNA ends



Examples of techniques used to detect DNA damage

1. Techniques involving complete DNA hydrolysis followed by determination of damaged entities by chromatography or mass spectrometry

HPLC: 8-oxo guanine determination

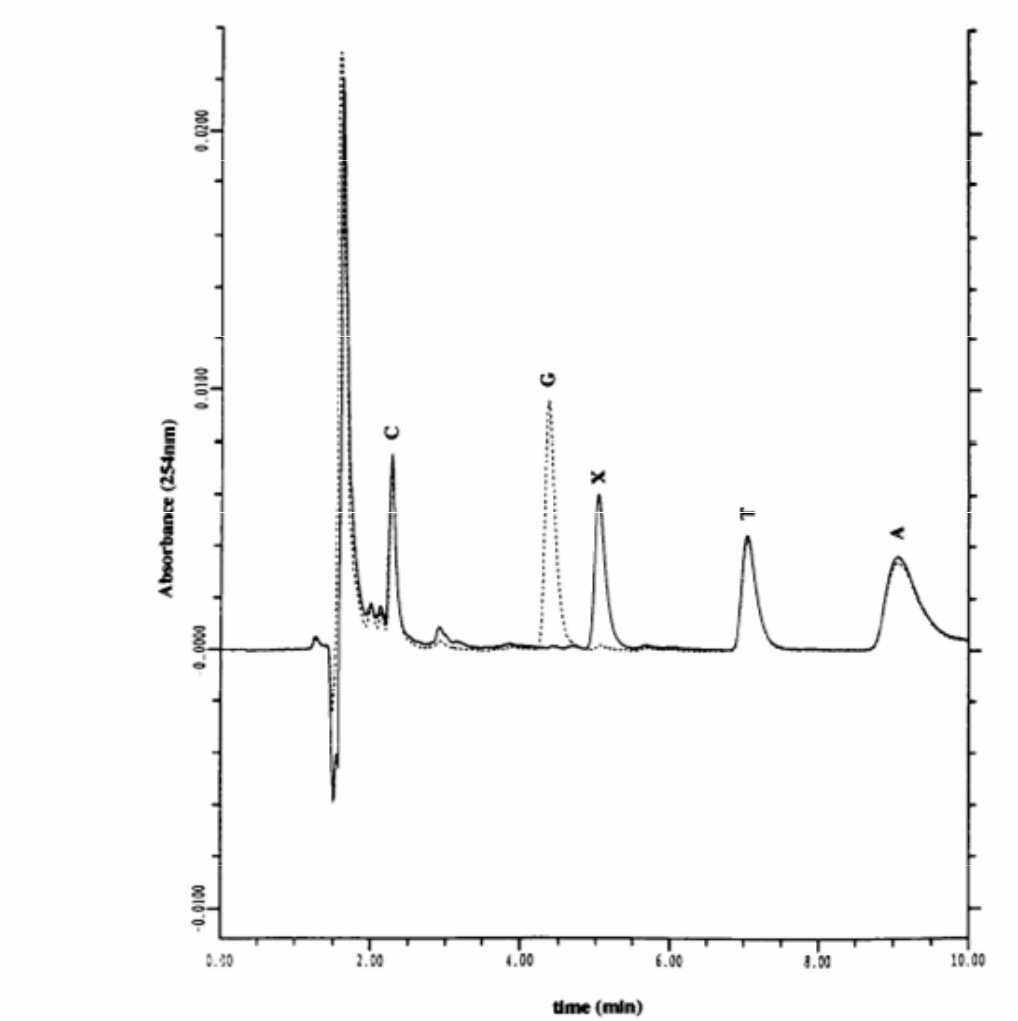


Fig. 4. Effect of guanase on bases derived from a formic acid hydrolysate of calf thymus DNA. Samples were HPLC with UV detection prior to (--) and following (—) guanase treatment as described in Materials and Methods. G, guanine; X, xanthine; T, thymine; A, adenine.

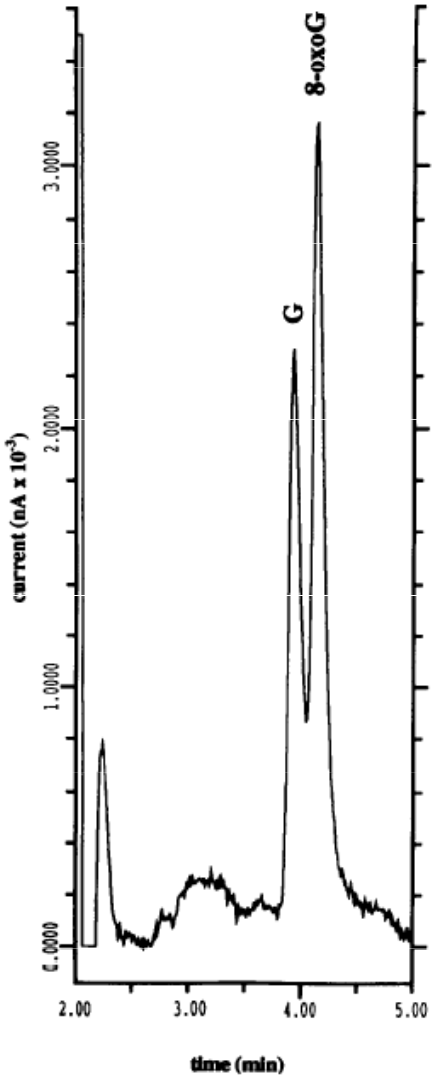
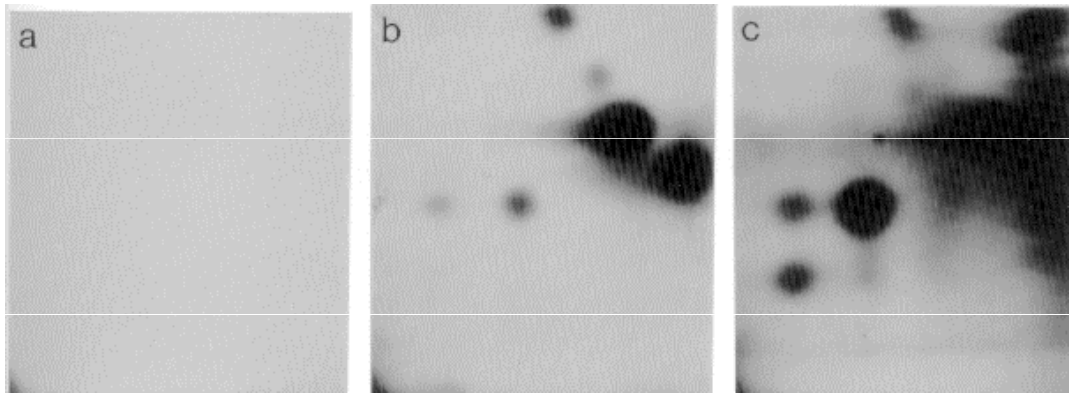


Fig. 1. Reversed-phase HPLC analysis, using electrochemical detection at +600 mV, of a solution containing 500 nM guanine (G) and 40 nM 8-oxoguanine (8-oxoG). Chromatographic conditions were as described in Materials and Methods except the mobile phase was 50 mM sodium acetate, 1 mM EDTA, pH5.1 containing 2% methanol.

^{32}P-postlabeling: analysis of base adducts



-Np-Np-Np-Np-Xp-Yp-Zp-
xenobiotic-modified DNA

↓

Enzymatic hydrolysis of DNA to 3'-mononucleotides using micrococcal endonuclease and spleen phophodiesterase

↓

Np + Np + Np + - - - + Xp + Yp + Zp + - -
Normal 3'-mononucleotides — Adducted 3'-mononucleotides

↓

Adduct enrichment by selective removal of normal 3'-mononucleotides
a) nuclease P1
b) n-butanol extraction

↓

Xp + Yp + Zp + - - - - -

↓

Radiolabel adducted 3'-mononucleotides with ^{32}P

↓

***pXp + *pYp + *pZp + - - - - -**

↓

Multidimensional thin-layer chromatography of adducts on PEI-cellulose

↓

Autoradiography or storage phosphor imaging to determine the distribution of radioactivity on the chromatograms

↓

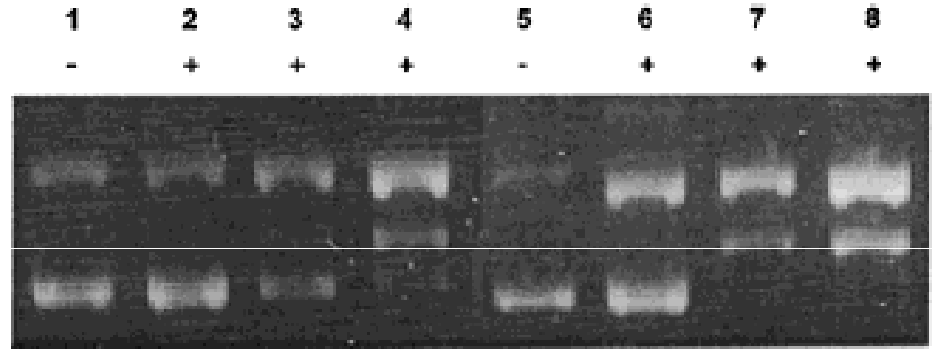
Quantitation of the radioactive areas on the chromatograms

1. Techniques involving complete DNA hydrolysis followed by determination of damaged entities by chromatography or mass spectrometry

2. Monitoring of changes in whole (unhydrolyzed) DNA molecules: electrophoretic and immunochemical techniques

detection of strand breaks:

relaxation (and/or linearization) of plasmid supercoiled DNA



scDNA (intact)

ocDNA (ssb)
linDNA (dsb) (damaged)

Figure 10. Relaxation of supercoiled pBR322 plasmid DNA by Fe(II)•deglycoBLM **11**. Lane 1, DNA + 10 μM deglycoBLM; lane 2, 1 μM deglycoBLM; lane 3, 5 μM deglycoBLM; lane 4, 10 μM deglycoBLM; lane 5, 10 μM deglycoBLM **11**; lane 6, 1 μM deglycoBLM **11**; lane 7, 5 μM deglycoBLM **11**; lane 8, 10 μM deglycoBLM **11**. Lanes 2–4 and 6–8 also contained 10 μM Fe^{2+}; essentially no cleavage was observed in the presence of Fe^{2+} alone.

„comet assay“ (dsb)

Fig.1 Unexposed control. Bundle of DNA (No-Tail)

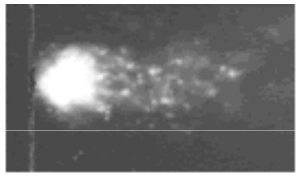


Fig.2 X-ray calibration 25.6 rads. DNA breaks are very obvious

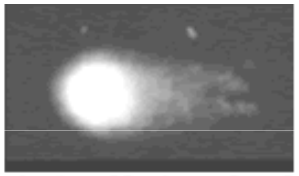
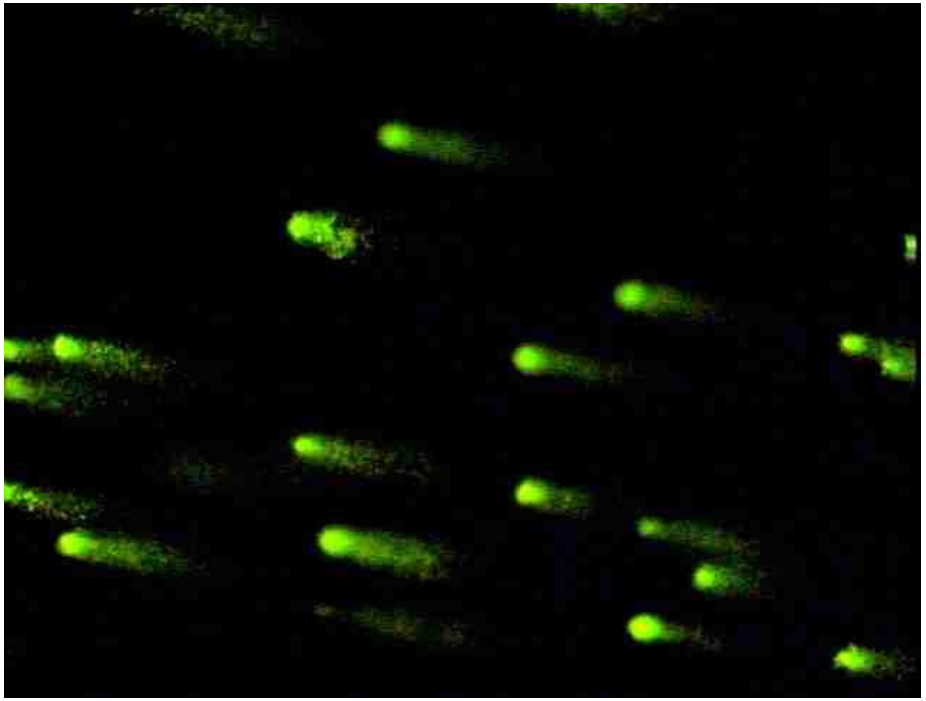


Fig.3 Cell Phone level microwave exposure 2hrs 2.45GHz reaching so called Safe SAR levels

Comet Tail = DNA Damage

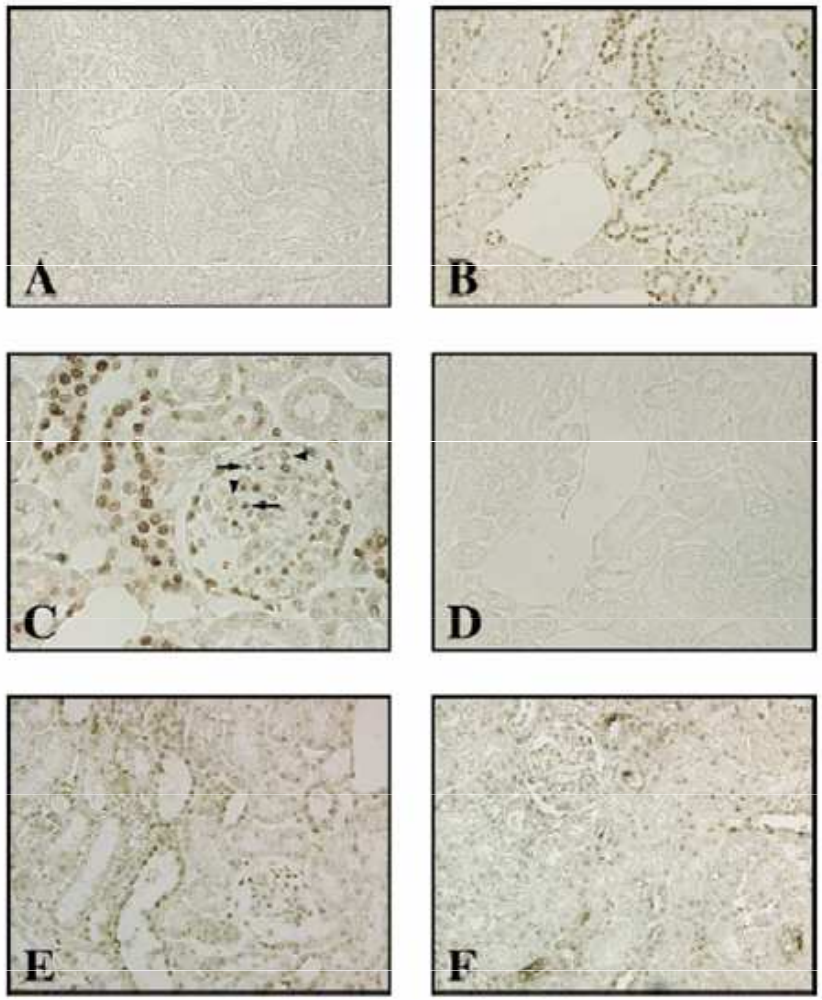
„alkaline elution assay“ (ssb + alkali-labile sites)



imunochemical techniques

when antibodies against the adducts available

- ELISA
- *In situ* techniques



8-oxo guanine detection *in situ* in kidney tissue